



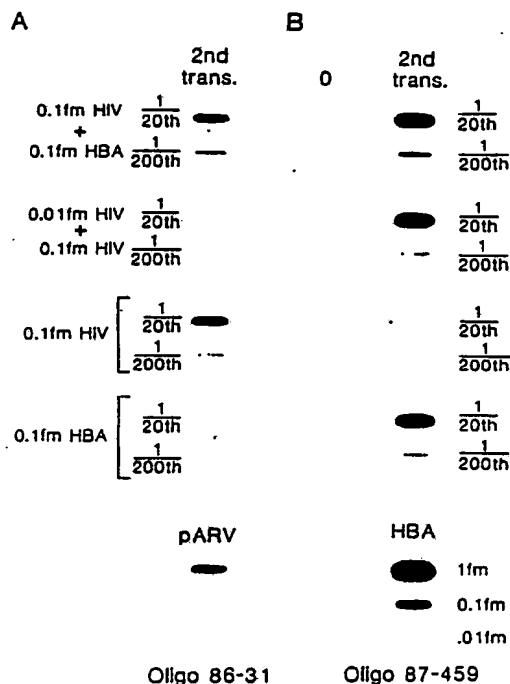
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(21) International Application Number: PCT/US88/02108 (22) International Filing Date: 17 June 1988 (17.06.88) (31) Priority Application Numbers: 064,141 202,978 (32) Priority Dates: 19 June 1987 (19.06.87) 6 June 1988 (06.06.88) (33) Priority Country: US (71) Applicant: SISKI DIAGNOSTICS, INC. [US/US]; 10280 North Torrey Pines Road, Suite 270, La Jolla, CA 92037 (US). (72) Inventors: GINGERAS, Thomas, Raymond ; 1528 Juniper Hill Drive, Encinitas, CA 92024 (US). MERTEN, Ulrich ; 4422 Leon Street, San Diego, CA 92107 (US). KWOH, Deborah, Yantis ; 2404 Jacaranda Avenue, Carlsbad, CA 92009 (US).		(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US). (81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU. Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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The present invention is predicated on the novelty of certain RNA transcripts, their production, optional replication, and use, to achieve desired amplification and detection of corresponding (in sequence) target nucleic acid sequence. The transcripts correspond in sequence to a target nucleic acid sequence contained in an original sample amongst a mixture of nucleic acids, and therefore, the presence of the transcripts in amplified form provides for their detection, and hence by correspondence, the *in vitro* or *x-vivo* detection of the presence of the target nucleic acid sequence in said sample.



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 More particularly, the present invention relates to novel methods and kits containing requisite reagents and means for increasing the in vitro or x-vivo copy number
10 of, i.e., amplifying, at least one selected segment (sequence) of nucleic acid or its complement or hybridizing homologous segment, in a sample comprising one or more nucleic acids, which may include RNA, DNA or both.

 Among the applications in which the methods and
15 kits of this invention find utility are 1) in analyses of body fluids and tissues for the detection of specific nucleic acid sequences characteristic of a genetic or pathogenic disease or condition by in vitro or x-vivo nucleic acid probe hybridization assays and 2) in the
20 selective cloning of single-copy or rare or low-expression genes.

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 Much of the work in molecular biology, molecular
25 genetics and applications thereof, such as for example, nucleic acid probe hybridization assays for blood-borne pathogens or defective genes, involves the detection or isolation of a particular nucleic acid sequence. A

fundamental problem in such work is to detect or isolate and then quantitate a nucleic acid sequence of interest. The problem has been a difficult one because biological materials, such as cell cultures, tissue specimens, and blood samples, typically are comprised of a complex mixture of RNA and DNA entities of which at most only a minuscule fraction has a sequence of interest.

Indeed, practical applications of nucleic acid probe hybridization assays have been limited because the sensitivity of the assays, when carried out with reagents suitable for routine use and over acceptably short time periods, is too low to detect sequences of interest at the low concentration at which they occur in real samples.

Two fundamentally different approaches have been taken to address the problem of detecting a nucleic acid sequence of interest ("target segment") present at a low level in a complex mixture of nucleic acids.

In the first approach, the amount of nucleic acid (including the target segment) in a sample is not altered; instead, a signal-generating system is associated with the target segment and produces a detectable signal representative of the number of molecules of target segment. For example, a nucleic acid probe, with a sequence complementary to that of a subsegment of the target segment is linked to an enzyme such as alkaline phosphatase and is mixed with sample under hybridization conditions that will effect hybridization between the probe and the target segment (but not appreciably between probe and other nucleic acid sequences in the sample). After removing enzyme-linked probe that failed to hybridize, a chromogenic substrate for alkaline phosphatase is added under suitable conditions and, in principle, a large number of detectable, colored molecules is rapidly produced for each probe molecule hybridized to target segment.

Numerous other systems for detecting nucleic acid sequences without altering the amount of target nucleic acid in the sample are known to the art. Another example is the commonplace labeling of a nucleic acid probe with radioactive atoms such as ^{32}P and then detecting probe hybridized to target via amplified signal initiated by decay of the radioactive nuclei. Still another example involves linking to probe for the target segment another nucleic acid that is capable of replication such that it can be readily detected by known techniques. Certain RNAs are known that are susceptible to (autocatalytically) replicase-induced replication by certain polymerase, such as bacteriophage RNA-dependent RNA polymerase, such as Q β replicase and the replicase from brome mosaic virus (BMV). In such a system, both an RNA and the RNA of complementary sequence are templates for replication by the RNA polymerase; consequently the amount of replicated RNA increases exponentially with time (as long as the number of RNA template molecules does not exceed the number of RNA polymerase molecules in a system). See Miele *et al.*, *J. Mol. Biol.* 171, 281 (1983). A system in which probe for a target segment is linked to an RNA capable of being replicated by Q β replicase is described by Chu *et al.*, *Nucl. Acids Res.* 14, 5591 (1986) and that by BMV replicase by Marsh *et al.*, *Positive Strand RNA Viruses*, Alan R. Liss (publr.; New York) (1987; Proceedings of UCLA Symposium, 1986).

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assay for a target segment, a signal due to "background" is unavoidable; that is, invariably there is some signal due to probe that non-specifically adheres to filters or other solid supports or hybridizes to segments with sequences closely similar to that of target segment. If the copy number of target is too low, the time constant ratio of signal from target plus background (i.e. "signal") to signal from background (i.e. "noise") will be too low to be detectable significantly above background. These and other drawbacks have led the art to a second approach of addressing the problem of detecting a target segment present at a low level in a complex mixture of nucleic acids.

This second approach is fundamentally different and involves increasing the copy number of the target segment itself, preferably to an extent greater than that of other sequences in a sample, particularly those that might erroneously be detected as target segment because of similarities in sequence. Examples of this second approach include various culture techniques in which cells that harbor the target segment are caused to increase in number, sometimes more rapidly than numbers of other cells, or in which particular nucleic acids (e.g., plasmids, RNAs) therein having disposed target segment are caused to increase in number.

Such culture techniques have the disadvantages of being cumbersome and problematic and time-consuming and manifest the inevitable: nucleic acids other than those which include target segment are simultaneously increased in copy number, thus potentially increasing "background." Another disadvantage is the resultant growth of potentially dangerous organisms as a necessary step to achieve amplification.

Another example of this second approach is amplification of a DNA target segment in a so-called "polymerase chain reaction" ("PCR"). This technique is a

borrowed adaptation of known, naturally occurring processes occurring in the replicative process of, for example, single-stranded DNA genomes of certain virus entities, and in all events, represents an application
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10 cycles, each of which entails (1) hybridizing to a 3'-terminal subsegment of each of the target segment and its complement (i.e., the segment of sequence complementary to that of target segment) a DNA primer, (2) extending each of the primers with a DNA polymerase, and
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Of course, it follows that this second approach can be applied to a sample in conjunction with use of the first approach applied with the amplified target segment to provide even a stronger detection signal.

5 It is an object of the present invention to solve the problems addressed by the prior art and to overcome the disadvantages enumerated in prior researchers endeavors to solve those problems. It is a further object of the present invention to provide a straightforward
10 technique that can be utilized in an acceptably short time, employing the convenience of known reagents and having the precision necessary to reach consistent scientific results; one that can be employed in a reproducible assay setting and that is adaptable for use
15 in kits for laboratory/clinical analyses.

It is thus an object of the present invention to increase the detectability of certain nucleic acid sequences (target segments) by amplification of said target sequences in an in vitro or x-vivo system devoid of
20 the disadvantages enumerated thus far by prior art endeavors.

The present invention is concerned with a novel technique for carrying out the second approach to detecting a target segment present at a low level in a
25 complex mixture of nucleic acids. It employs a novel RNA transcript production step in conjunction with, and derived from, a synthesized double-stranded cDNA copy of the target sequence as a complete cycle. Multiple cycles can be employed. By virtue of the transcription step
30 being the dominant aspect of novelty, it is conveniently referred to herein as a transcription-based amplification system (TAS). The novel technique of the present TAS invention results in rapid increase in copy number of a selected target segment by making use of two properties of
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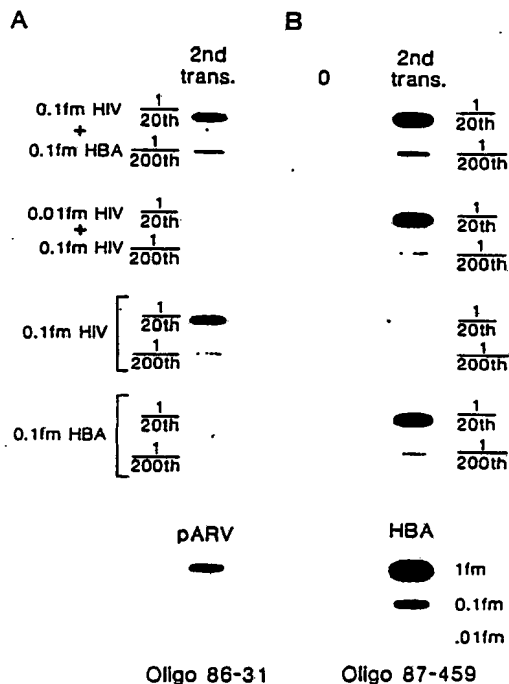
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The present invention is concerned with a novel technique for carrying out the second approach to detecting a target segment present at a low level in a
25 complex mixture of nucleic acids. It employs a novel RNA transcript production step in conjunction with, and derived from, a synthesized double-stranded cDNA copy of the target sequence as a complete cycle. Multiple cycles can be employed. By virtue of the transcription step
30 being the dominant aspect of novelty, it is conveniently referred to herein as a transcription-based amplification system (TAS). The novel technique of the present TAS invention results in rapid increase in copy number of a selected target segment by making use of two properties of
35 DNA-dependent RNA polymerase: (1) appreciable initiation of transcription from only a small number of sequences

specific for each polymerase, see, e.g., Brown et al.,
Nucl. Acids Res. 14, 3521 (1986); and (2) rapid production
of a large number of transcripts from each copy of a
promoter (typically 10^2 - 10^4 per hour) recognized by an RNA
polymerase. See Milligan et al., Nucleic Acids Res. 15,
8783 (1987). The technique of the invention can also
utilize the ability of RNAs with certain sequences to be
rapidly (autocatalytically) replicated by RNA-dependent
RNA replicases. See also Miele et al., supra. In
addition, it provides a standardization technique making
possible unambiguous measurement of the amount of target
DNA present in a sample.

The present invention (unless induced
(autocatalytic) replication is employed) yields a
single-stranded RNA transcript, or an RNA-DNA duplex
formed therefrom when measures are not undertaken to
prevent its formation, that has a subsegment that has the
sequence of the target segment or the sequence
complementary to that of the target segment, and that is
present in large excess relative to nucleic acid with a
subsegment of complementary sequence. This excess
initially of a single-stranded RNA transcript is
advantageous in certain methods for detecting amplified
product with a labeled nucleic acid probe because little
segment of complementary sequence is present to compete
with probe for hybridizing to the amplified product.
Also, the single-stranded RNA transcript product hereof is
struck-off more or less continuously and provides for
direct detection of target segment without the necessity
of cumbersome, error-prone repeated PCR cycles and strand
separation. Such advantages are not provided by the PCR
technique that yields double-stranded DNA (one strand of
which comprises target segment and the other strand of
which comprises complement of target segment) that need to
be separated before detection and only after a large

number of repeated cycles necessary to reach acceptable amplification levels.

5 The techniques of the present invention provide amplification of a selected target segment to an extent at least as great as the PCR technique over about the same period of time, but in a far more simplistic and reproducible manner and distinct from natural processes or other art.

Summary of the Invention

10 We have discovered how bacteriophage DNA-dependent RNA polymerase can be used to rapidly amplify (i.e., increase the copy number of) a selected target nucleic acid sequence (target sequence or segment) present in a sample of nucleic acids. Further, we have discovered
15 how bacteriophage DNA-dependent RNA polymerase can be used together with bacteriophage RNA-dependent RNA polymerase to accomplish the same result. Heretofore, it has not been appreciated that such bacteriophage RNA polymerase could be used for this purpose.

20 The invention entails methods based on these discoveries and kits for carrying out the methods.

 These methods and kits are particularly usefully applied in connection with nucleic acid probe hybridization assays for a nucleic acid which includes a
25 target segment amplified in accordance with the invention. Thus, the invention also entails methods, and kits for carrying out the methods, for detecting the presence or absence of a segment in a sample of a nucleic acid, which comprises a particular segment, by means of a probe
30 hybridized to that segment after amplification in accordance with the invention.

 The present invention is predicated on the novelty of certain RNA transcripts, their production, optional replication, and use, to achieve desired amplification and

detection of corresponding (in sequence) target nucleic acid sequence. The invention is practiced in an in vitro or x-vivo setting, and is employed conjunctively with the synthesis of a double-stranded cDNA copy of the target sequence in order to produce a double-stranded nucleic acid template used in turn for production of said RNA transcripts. This process of double-stranded cDNA synthesis and RNA transcription constitutes a single cycle of the present transcription-based amplification system (TAS). If desired, this cycle may be repeated in order to achieve even higher levels of amplification. By virtue of the method by which they are produced (and reproduced), the transcripts correspond (identically or complementarily) in sequence to a target nucleic acid sequence contained in an original sample amongst a mixture of nucleic acids, and therefore, the presence of the transcripts in amplified form provides for their detection, and hence by correspondence, the in vitro or x-vivo detection of the presence of the target nucleic acid sequence in said sample.

Thus, the present invention involves the in vitro or x-vivo detection of at least one specific nucleic acid sequence (target sequence or segment) in a sample containing nucleic acid. The present invention reduces to a method comprising preparing a double-stranded nucleic acid containing a sequence corresponding to a target sequence operably linked to a promoter therefor, employing said double-stranded nucleic acid as a double-stranded nucleic acid template for the preparation of a plurality of RNA transcripts therefrom, each bearing an RNA sequence corresponding to said target sequence, and detecting the presence of said RNA sequence and by analogy the presence of target sequence.

The present invention is directed to all methods and means associated with the preparation and use of such RNA transcripts. Thus, the present invention is directed

to the optionally repetitive method of preparing said double-stranded nucleic acid template defined above comprising providing a first nucleic acid primer containing a promoter sequence operably linked to a
5 sequence corresponding to a segment of a target sequence, as defined above, hybridizing under suitable conditions said first nucleic acid primer with target sequence in a sample containing nucleic acid, extending the hybridized said first nucleic acid primer in a polymerase extension
10 reaction complementarily to the target sequence to form a corresponding duplex nucleic acid, separating the strands of said duplex, hybridizing to the separated promoter containing sequence strand under suitable conditions a second nucleic acid primer at the end opposite said
15 promoter sequence, and extending the hybridized said second nucleic acid primer in a polymerase extension reaction complementarily to said promoter containing sequence.

The present invention is further directed to
20 further, and alternative, methods and means of preparing said double-stranded nucleic acid template (supra), for example, an essentially single-pot reaction comprising providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence
25 complementary to a segment of a target sequence and a second nucleic acid primer having a sequence identical to a segment of a target sequence, said primers corresponding to different regions of said target sequence but not, or not substantially, overlapping in their correspondence
30 to the target and being selected such that an extension product of one when separated from its complement can serve as a template for an extension product of the other, contacting a sample containing nucleic acid including target sequence with said primers under sequential
35 hybridizing and strand separation conditions so as to produce, in turn, extension products of said primers.

The present invention is further directed to methods and means of employing said double-stranded nucleic acid supra., as a template for the preparation of a plurality of RNA transcripts therefrom in a reaction catalyzed by a DNA-dependent RNA polymerase that recognizes the promoter thereof, and detecting and measuring, the presence of said RNA transcripts.

The present invention is further directed to methods and means to standardize the amount of target sequence detected (by correspondence to the amount of RNA transcript detected) by further correlation to the presence of known nucleic acid used as an internal standard. The copy number of the standard is predetermined, the standard shall experience the same conditions, hence fate, during the practice of this invention as does target sequence and it shall therefore serve as an evaluation means in determining relative amounts of transcripts prepared in parallel for it and for target sequence.

The present invention is further directed to the further replication of obtained RNA transcripts defined above via the presence therein of replicase recognition sites. This is conveniently accomplished by providing said first nucleic acid primer defined above additionally bearing a replicase recognition site sequence, preferably between the promoter sequence and sequence corresponding to target sequence, and/or additionally containing a replicase recognition site on the second nucleic acid primer defined above. On subsequent transcription, the consequent transcripts bear the replicase recognition site(s), such that presence of a replicase (in the reaction locus or kit for example) (autocatalytically) induces replication of the transcripts producing additional copies to further facilitate detection.

The present invention is further directed to kits comprising requisite reagents and associated means useful

in the in vitro or x-vivo detection of at least one specific nucleic acid sequence (target sequence) in a sample containing nucleic acid, employing the methods and means defined supra.

5 Detailed Description of the Invention

1. Brief Description of the Drawings

Figures 1A, 1B and 1C illustrate a method according to the invention for amplifying a target segment of a nucleic acid (Nucleic Acid A), which is a DNA or RNA,
10 wherein many copies of a first RNA (RNA I) with a segment with a sequence complementary to that of target segment are made.

Figures 2A, 2B, 2C illustrate the further amplification according to the invention of a segment of
15 an RNA I, made as illustrated in Figures 1A, 1B and 1C, to make many copies of a second RNA (RNA II) with a segment with a sequence the same as that of a subsegment of the target segment, which was amplified to make the RNA I.

Figure 3 depicts autoradiograms showing various
20 concentrations of HIV RNA amplified by TAS simultaneously with a fixed concentration of human β -globin nucleic acid.

Figure 4 displays a general strategy whereby RNA produced through a DNA-dependent RNA polymerase yields an
25 RNA molecule that can serve as a template for an RNA-dependent replicase.

2. General Methods and Definitions

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques of the present
30 invention such as: DNA probe or primer preparation, including DNA synthesis; hybridization methodology

including variations in stringency conditions for producing more or less hybridization certainty depending upon the degree of homology of the primer to a target DNA sequence; identification, isolation or preparation of promoters, or more specifically promoters or sites recognized by bacteriophage DNA-dependent RNA polymerase and bacteriophage RNA-dependent RNA polymerase, or in the employment of eukaryotic systems, viral DNA- and RNA-dependent RNA polymerases, for example, adenovirus encoded RNA polymerase and brome mosaic virus RNA polymerase; conditions conducive to the production of RNA transcripts, including so-called transcription enhancer sequences; the mechanism and methodology for (induced) replication; polymerase chain reaction methods including the reagents used therein; and so forth. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, (1982), and the various references cited therein; U.S. Patent 4683195; U.S. Patent 4683202; Hong, Bioscience Reports 1, 243 (1981); Cooke et al., J. Biol. Chem. 255, 6502 (1980); and Zoller et al., Methods in Enzymology 100, 468-500 (1983); Crea et al., Nucleic Acids Res. 8, 2331 (1980); Narang et al., Meth. Enzym. 68, 90 (1979); Beaucage et al., Tetrahedron Letters 22, 1859 (1981); Brown et al., Meth. Enzym. 68, 109 (1979); Caruthers et al., Meth. Enzym. 154, 287 (1985); Hitzeman et al., J. Biol. Chem. 255, 2073 (1980); Lee et al., Science 239, 1288 (1988); Milligan et al., Nucleic Acids Res. 15, 8783 (1987); Miller et al., Virology 125, 236 (1983), Ahlquist et al., J. Mol. Biol. 153, 23 (1981); Miller et al., Nature 313, 68 (1985); Ahlquist et al., J. Mol. Biol. 172, 369 (1984); Ahlquist et al., Plant Mol. Biol. 3, 37 (1984); Ou et al., PNAS 79, 5235 (1982); Chu et al., Nucl. Acids Res. 14, 5591 (1986); European Patent Application Publ'n. No. (EPA) 194809; Marsh et al., Positive Strand RNA Viruses, p. 327-336, Alan R. Liss (publ.; New York) (1987; Proceedings of UCLA

Symposium, 1986); Miller et al., J. Mol. Biol. 187, 537 (1986); Stoflet et al., Science 239, 491 (1988); and Murakawa et al., DNA 7, 287 (1988).

5 All of the aforecited publications are by this reference hereby incorporated by reference herein.

By the term "promoter" is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that is specifically recognized by an RNA polymerase that binds to a recognized
10 sequence and initiates the process of transcription whereby an RNA transcript is produced. It may optionally contain nucleotide bases extending beyond the actual recognition site, thought to impart additional stability toward degradation processes. In principle, any promoter
15 sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Typical, known and useful promoters are those that are recognized by certain bacteriophage polymerase such as bacteriophage T3, T7 or SP6. See
20 Siebenlist et al., Cell 20, 269 (1980). These are but examples of those polymerase which can be employed in the practice of the present invention in conjunction with their associated promoter sequences.

The "RNA transcript" hereof is the ribonucleic
25 acid sequence produced after transcription initiation following RNA polymerase recognition of the promoter sequence (See supra). The production of such transcripts is more or less continuous, dependent in part on the amount of polymerase present.

30 By the term "primer" in the present context is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that has sufficient homology with the target sequence such that under suitable hybridization conditions it is capable
35 of hybridizing, that is binding to, the target sequence. A typical primer is at least about 10 nucleotides in

length, and most preferably is of approximately 35 or more nucleotide bases in length, and in its most preferred embodiments, it shares identity or very high homology with the target sequence. See, for example, EPA 128042 (publd. 12 Dec 84).

The term "operably linked" in particular in connection with the linkage of a promoter sequence within a primer sequence, refers to the functionality of the ultimate "double-stranded nucleic acid template" of the present invention such that it, the template, is capable of producing corresponding RNA transcripts when the promoter is recognized by the suitable polymerase--see supra.

The primer extension reaction to produce a duplex is known per se. See references supra. Polymerase useful for this purpose include E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, reverse transcriptase, and so forth.

The techniques of forming a detection signal such as via radioactive labeling or chromogenic means using a chromogenic susceptible enzyme are also well known and documented in the art. See discussion supra.

The use of a "replicase" for (autocatalytic) induction of replication of the RNA transcripts of the present invention are generally known in the art. Suitable examples of such replicases that are useful in the present invention include the so-called Q β virus replicase that recognizes certain nucleic acid sequence sites at both the 3'- and 5'- ends of the given RNA transcript and the so-called brome mosaic virus (BMV) as well as the alpha virus replicases which are thought to recognize nucleic acid sequence sites at the 3'- end of a given RNA transcript. These replicases serve to replicate, that is reproduce, the RNA transcripts and complements so as to multiply copies thereof. When such enzyme is present in the reaction locus during the process

of transcription, it can be foreseen that the multiple transcripts that are produced during transcription can themselves undergo replication so as to exponentially increase the amount of RNA transcript product.

5 Internal standardization is defined as a process which is used to: a) insure that the TAS amplification process has not failed because of a procedural error, and b) measure the levels of target nucleic acid relative to a
10 predetermined quantity of nucleic acid which is always associated with the sample of interest. Such internal standardization occurs by coamplifying a portion of the target sequence, as well as an endogenous sequence, in the same reaction. For example, by knowing the cell count
15 present within the biological sample, a single-copy gene (e.g., β -globin) could be used as an internal standard and, since it is not expressed in the form of RNA, its initial copy number is equal to two times the total number of cells in the sample. By coamplifying portions of the
20 β -globin and target sequences of interest, the ratios of the amplified signals can be compared to quantitate the levels of target sequence of interest. Since every cell has two copies (in diploid cells) of this internal standard, irrespective of whether the biological sample contains separate target sequences (e.g., HIV), each
25 sample is expected to produce a positive amplification signal resulting from the internal standard. See Groudine et al., Nucleic Acids Research 12, 1427 (1984) and McKnight, Cell 31, 355 (1982). See also British Patent Application Publn. No. 2187283 A (publd. 3 Sept. 87).

30 3. Detailed Description of Preferred Aspects

In one of its aspects, the invention is a method for amplifying a target nucleic acid segment of Formula I

3'-(first subsegment)_t-(second subsegment)_t-

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(third subsegment)_t-5'

I

wherein (first subsegment)_t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 3'-terminus of (second subsegment)_t, (second subsegment)_t is a nucleic acid segment of 0 or more nucleotides, and (third subsegment)_t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 5'-terminus of (second subsegment)_t, which method comprises:

(1) hybridizing to (first subsegment)_t of said target segment a first primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula II

5'-(promoter)₁-(variable subsegment)₁-(3'-primer subsegment)₁-3'

II

wherein (promoter)₁ is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage DNA-dependent RNA polymerase-specific promoter, (variable subsegment)₁ is a single-stranded DNA segment of 0 to 100 nucleotides adjoining the 3'-terminal nucleotide of (promoter)₁ and the 5'-terminal nucleotide of (3'-primer subsegment)₁, and (3'-primer subsegment)₁ is a single-stranded DNA segment of at least 10 nucleotides with a sequence which is complementary to the sequence of a subsegment of (first subsegment)_t which terminates with the 3'-terminal nucleotide of (first subsegment)_t, said (promoter)₁ adjoining the 5'-terminus of said (3'-primer subsegment)₁, if said (variable subsegment)₁ has 0 nucleotides;

(2) extending said first primer, hybridized in accordance with step (1), in a reaction catalyzed by a

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first DNA polymerase to make a first complementary DNA segment, which comprises a subsegment of Formula III

5'- (promoter)₁- (variable subsegment)₁-
(first subsegment)_{tc}- (second subsegment)_{tc}-
(third subsegment)_{tc}-3',

III

wherein (first subsegment)_{tc} is the DNA segment with the sequence complementary to that of (first subsegment)_t, (second subsegment)_{tc} is the DNA segment with the sequence complementary to that of (second subsegment)_t, and (third subsegment)_{tc} is the DNA segment with the sequence complementary to that of (third subsegment)_t, provided that, if the target segment is an RNA segment, said first DNA polymerase is a reverse transcriptase;

(3) rendering single-stranded the duplex formed in the reaction of step (2);

(4) hybridizing to (third subsegment)_{tc} of said first complementary DNA of Formula III a second primer, which is a single-stranded DNA of at least 10 nucleotides of Formula IV 3'-(5'-primer subsegment)₂-(variable subsegment)₂-5',

IV

wherein (5'-primer subsegment)₂ has the sequence of a subsegment of (third subsegment)_t which terminates with the 5'-terminal nucleotide of (third subsegment)_t and wherein (variable subsegment)₂ is a segment of 0 to 100 nucleotides which adjoins the 5'-terminus of (5'-primer subsegment)₂;

(5) extending said second primer segment, hybridized in accordance with step (4), in a reaction catalyzed by a second DNA polymerase to form a second

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complementary DNA segment which comprises a subsegment of Formula V

5'-(variable subsegment)₂-(third subsegment)_t-
 (second subsegment)_t-(first subsegment)_t-
 5 (variable subsegment)_{1c}-(promoter)_{1c}-3',

V

wherein (variable subsegment)_{1c} is the DNA segment with the sequence complementary to that of (variable subsegment)₁, and (promoter)_{1c} is the DNA segment with the sequence complementary to that of (promoter)₁, provided that said second DNA polymerase is the same as or different from said first DNA polymerase; and

(6) employing the double-stranded product of step
 15 (5) as the template in a reaction catalyzed by a first bacteriophage DNA-dependent RNA polymerase, which recognizes the promoter of which one strand is (promoter)₁, to make a first RNA product of Formula VI
 5'-(variable subsegment)_{1r}-(first subsegment)_{tcr}-
 20 (second subsegment)_{tcr}-(third subsegment)_{tcr}-
 (variable subsegment)_{2cr}-3'

VI

wherein (variable subsegment)_{1r} is the RNA segment with the sequence of (variable subsegment)₁, (first subsegment)_{tcr} is the RNA segment with the sequence complementary to that of (first subsegment)_t,
 25 (second subsegment)_{tcr} is the RNA segment with the sequence complementary to that of (second subsegment)_t,
 (third subsegment)_{tcr} is the RNA segment with the sequence complementary to that of (third subsegment)_t, and
 30 (variable subsegment)_{2cr} is the RNA segment with the sequence complementary to that of (variable subsegment)₂.

In another of its aspects, the invention entails a

20

method wherein (first subsegment)_t is at least 10 nucleotides in length and is of Formula XIII

3'-(first subsegment)_{t2}-(first subsegment)_{t1}-5',

XIII

5 wherein (first subsegment)_{t2} has 0 or more nucleotides and, if more than 0, adjoins the 3'-terminus of (first subsegment)_{t1}, and (first subsegment)_{t1} is at least 10 nucleotides in length; wherein (third subsegment)_t is of Formula XIV

10 3'-(third subsegment)_{t1}-(third subsegment)_{t2}-5,

XIV

wherein (third subsegment)_{t2} has 0 or more nucleotides and, if more than 0, adjoins the 5'-terminus of (third subsegment)_{t1}, and (third subsegment)_{t1} is at least 10 nucleotides in length; wherein (3'-primer subsegment)₁ has the sequence complementary to that of a subsegment of target segment which consists of all of (first subsegment)_{t2} and 0 or more nucleotides of (first subsegment)_{t1}; wherein (5'-primer subsegment)₂ is a subsegment which consists of all of (third subsegment)_{t2} and 0 or more nucleotides of (third subsegment)_{t1}; and wherein, after steps (1) to (6) Supra, the RNA subsegment of Formula VII

25 5'-(first subsegment)_{t1cr}-(second subsegment)_{tcr}-(third subsegment)_{t1cr}-3'

VII

wherein (first subsegment)_{t1cr} is the RNA segment with the sequence complementary to that of (first subsegment)_{t1} and (third subsegment)_{t1cr} is the RNA segment with the sequence complementary to that of (third subsegment)_{t1}, is further amplified by a method which comprises:

(7) hybridizing to said first RNA product of Formula VI a third primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula VIII

5'- (promoter)₃- (variable subsegment)₃-
 5 (3'-primer subsegment)₃-3',

VIII

wherein (promoter)₃ is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage DNA-dependent RNA polymerase-specific promoter, said
 10 sequence of (promoter)₃ being the same as or different from that of (promoter)₁, (variable subsegment)₃ is a single-stranded DNA segment of 0 to 100 nucleotides which adjoins the 3'-terminal nucleotide of (promoter)₃ and the
 15 (3'-primer subsegment)₃ is a single-stranded DNA segment which has the same sequence as (third subsegment)_{t1} and adjoins the 3'-terminal nucleotide of (promoter)₃ if (variable subsegment)₃ has 0 nucleotides;

(8) extending said third primer hybridized in
 20 accordance with step (7) in a reaction catalyzed by a third DNA polymerase, which is a reverse transcriptase and is the same as or different from said first and second DNA polymerase, to make a third complementary DNA segment which comprises a 3'-terminal subsegment of Formula IX

25 5'- (promoter)₃- (variable subsegment)₃-
 (third subsegment)_{t1}- (second subsegment)_t-
 (first subsegment)_{t1}- (first subsegment)_{t2}-
 (variable subsegment)_{1c}-3',

IX

30 wherein (variable subsegment)_{1c} is the DNA with the sequence complementary to that of (variable subsegment)₁;

22

(9) rendering single-stranded the duplex formed in the reaction of step (8);

(10) hybridizing to the third complementary DNA made in the reaction of step (8) a fourth primer of
5 Formula X

5'-(variable subsegment)₄-(5'-primer subsegment)₄

X

wherein (variable subsegment)₄ is a segment of 0 to 100 nucleotides, and (5'-primer subsegment)₄ is a subsegment
10 of known sequence which adjoins the 3'-nucleotide of (variable subsegment)₄, if (variable subsegment)₄ has more than 0 nucleotides, and which comprises at least 10 nucleotides of the segment of Formula XX

5'-(variable subsegment)₁-(first subsegment)_{t2c}-(first
15 subsegment)_{t1c}-3',

XX

wherein (first subsegment)_{t2c} is the DNA segment with the sequence complementary to that of (first subsegment)_{t2} and (first subsegment)_{t1c} is the DNA segment with the sequence
20 complementary to that of (first subsegment)_{t1}, provided that at least one of said at least 10 nucleotides is at or 5' from the 5'-terminal nucleotide of (first subsegment)_{t1c};

(11) extending said fourth primer hybridized in
25 accordance with step (10) in a reaction catalyzed by a fourth DNA polymerase, which is the same as or different from said first, second and third DNA polymerase, to form a fourth complementary DNA which comprises a segment of Formula XI

23

5'-(first subsegment)_{t1c}-(second segment)_{tC}-(third subsegment)_{t1c}-(variable subsegment)_{3c}-(promoter)_{3c}-3',

XI

wherein (second subsegment)_{tC} is the DNA segment with the sequence complementary to that of (second subsegment)_t,
 5 (third subsegment)_{t1c} is the DNA segment with the sequence complementary to that of (third subsegment)_{t1}, (variable subsegment)_{3c} is the DNA segment with the sequence complementary to that of (variable segment)₃, and
 10 (promoter)_{3c} is the DNA segment with the sequence complementary to that of (promoter)₃; and

(12) employing the double-stranded product of step (11) as the template in a reaction catalyzed by a second bacteriophage DNA-dependent RNA polymerase, which is the
 15 same as or different from said first bacteriophage DNA-dependent RNA polymerase and which recognizes the promoter of which one strand is (promoter)₃, to make a second RNA product with a 5'-terminal subsegment of Formula XII 5'-(variable subsegment)_{3r}-(third
 20 subsegment)_{t1r}-(second subsegment)_{tr}-(first subsegment)_{t1r}-X₁₂-(variable subsegment)_{4cr}-3',

XII

wherein (variable subsegment)_{3r} is the RNA segment with the sequence of (variable subsegment)₃, (third
 25 subsegment)_{t1r} is the RNA segment with the sequence of (third subsegment)_{t1}, (second subsegment)_{tr} is the RNA segment with the sequence of (second subsegment)_t, (first subsegment)_{t1r} is the RNA segment with the sequence of
 (first subsegment)_{t1}, (variable subsegment)_{4cr} is the RNA
 30 segment with the sequence complementary to that of (variable subsegment)₄, and X₁₂ is the RNA segment with the sequence complementary to that of the subsegment of (5'-primer subsegment)₄ that is 5' from the 5'-terminus of (first subsegment)_{t1c}.

As noted supra, the invention also entails kits for carrying out the amplification methods of the invention. In the case of the method of the invention which entails making a first RNA product, a kit of the invention would comprise the two primers, the
5 corresponding bacteriophage DNA-dependent RNA polymerase, and the DNA polymerase. A kit for carrying out a method in accordance with the invention which entails making both a first and a second RNA product would comprise four
10 suitable primers (two sets of two) with the corresponding necessary polymerases.

Methods and kits according to the invention for carrying out nucleic acid hybridization probe assays which entail amplifying a target segment in accordance with the
15 invention entail, in addition to the steps and components, respectively, of the methods and kits for amplification, steps and components necessary for detecting the RNA product resulting from amplification according to the invention. The skilled understand the various additional
20 steps and components, respectively, that are required to detect RNA from an amplification process by any of the numerous nucleic acid probe hybridization assay methods known in the art. One preferred nucleic acid probe hybridization assay method, involving bead-capture of
25 labeled RNA amplification, is illustrated in Example II below.

It is preferred that (3'-primer subsegment)₁, (5'-primer subsegment)₂, (3'-primer subsegment)₃, and (5'-primer subsegment)₄ have between 20 and 40
30 nucleotides, and more preferably about 30 nucleotides to enhance the specificity with which the various primers bind to the ends of the target segments which are sought to be amplified.

It is also preferred that the target segment of a
35 nucleic acid of interest which is selected for amplification (by virtue of selection of (first

subsegment)_t and (third subsegment)_t be selected so that (second subsegment)_{tr} have at least 30, and more preferably at least about 50, nucleotides. This permits the use of (second subsegment)_{tc} or (second subsegment)_{tr} in the amplified RNA product to be targets for nucleic acid probes that will not have sequences that overlap those of any of the primer subsegments.

While the bacteriophage DNA-dependent RNA polymerase can use templates longer than 1000 bp, their efficiency of transcription decreases as the template becomes longer. Thus, target segments less than 1000 bases long are favored.

It is preferred to use the identical set of primers used in the synthesis of RNA, to produce RNA in a second cycle of TAS. As stated in the section describing the production of RNA, the primer pair should not overlap. In carrying out the method of the invention in which the production of RNA is desired, it is possible that the subsegment of target segment with sequence complementary to that of (5'-primer subsegment)₄ be in the 5'-direction from and not overlap the subsegment of target segment with sequence complementary to that of (3'-primer subsegment)₁. Similarly it is preferred that the subsegment of target segment with sequence the same as that of (3'-primer subsegment)₃ be in the 3'-direction from and not overlap the subsegment of target segment with sequence the same as that of (5'-primer subsegment)₂. This strategy may be preferred because it reduces the competition of the different primers for the same sites and, thereby, may markedly enhance the efficiency of amplification according to the invention. Where there is some overlap between sets of nested primers, it is preferable to remove any unused first set of primers, before employing the second set.

The focus of the present invention is on bacteriophage DNA-dependent RNA polymerase because of

their high specificity for certain promoters. Other polymerase which have similarly high specificity for particular promoters could be employed in accordance with the invention in place of the bacteriophage polymerase and the invention is intended to cover such other polymerase as well.

The preferred of the bacteriophage DNA-dependent RNA polymerase are those from T7, T3 and SP6. Preferred promoters for use with these polymerase are described in the examples and claims, but numerous other promoters for these polymerase are known in the art and can be employed as well.

Further, polymerase from bacteriophages other than the preferred three, and promoters recognized by such other polymerase, can be employed in accordance with the invention.

It is preferred to employ for DNA polymerase in the methods of the invention reverse transcriptases and DNA polymerase which lack 5' to 3' exonuclease activity. It is most preferred that a single DNA polymerase be used for all steps in the methods. Most preferred of the DNA polymerase are the AMV reverse transcriptase and recombinant MMLV reverse transcriptase. Other polymerase are acceptable however, such as the heat-stable DNA polymerase from *Thermus aquaticus* (see Chien et al. *J. Bacteriol.* 127, 1550 (1976)), SequenaseTM brand recombinant T7 DNA polymerase from the U. S. Biochemicals Corp., Cleveland, Ohio, U.S.A., and the well known Klenow Fragment of *E. coli* DNA polymerase I, and calf thymus DNA polymerase alpha.

The "variable subsegments" that are optionally included in the various primers serve three functions. In fact, they might be more aptly termed "multipurpose subsegments." First, for the first and third primers, which include promoters, the promoter subsegments preferably include transcription initiation sequences that

are preferred by the RNA polymerase corresponding to the promoter. Second, for all of the primers, a variable subsegment can optionally contain a particular segment whereby RNA product from the amplification can be detected in a nucleic acid probe hybridization assay. Indeed, amplification (and assay) can occur for several different target segments simultaneously by using sets of primers that differ in their recognition segments (e.g., (3'-primer subsegment)₁) but include a common variable subsegment. The variable subsegment can also contain a polylinker sequence that conveniently contains a plurality of restriction sites for ease of cloning subsequently. Finally, the variable subsegments can be used to incorporate into the RNA product from amplification sequences necessary to allow the RNA product to be (autocatalytically) replicated by a bacteriophage RNA-specific RNA polymerase, such as the Q β replicase, see Miele *et al.*, *supra*, and BMV sequences from the RNA-3 chromosome of the plant virus that promotes the synthesis of coat mRNA. See Ahlquist *et al.*, *J. Mol. Biol.* 172, 369 (1984); Ahlquist *et al.*, *Plant Mol. Biol.* 3, 37 (1984); Ahlquist *et al.*, *J. Mol. Biol.* 153, 23 (1981); Miller, *et al.*, *Nature* 313, 68 (1985); Miller *et al.*, *Virology* 125, 236 (1983); and Ou *et al.*, *PNAS* 79, 5235 (1982).

In the case of the Q β replicase, which is known in the art, this is preferably carried out by including in (variable subsegment)₂ the sequence 5'-CGCGCTCTCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3' and in (variable subsegment)₁ the sequence 5'-TGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3', and then (autocatalytically) replicating the first RNA product (i.e., RNA. I in Figure 1; see also Figure 4), or including in (variable subsegment)₄ the sequence 5'-CGCGCTCTCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3' and in (variable subsegment)₃ the sequence 5'-TGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3', and then

(autocatalytically) replicating the second RNA product (i.e., RNA II in Figure 1; see also Figure 4).

In the case of BMV replicase use, also known, this is preferably carried out by including the following sequence for BMV in variable sequence 2 (bases 1-25) (in the specific use of the HIV example infra):

BMV promoter

TCS = 87-29 (HIV-specific)

10 5'-AAGATCTATGTCCTAATTCAGCGTA|ACAGCATATGTATGTTTCAGGGA

15 AAGCTA-3' (54 bases), as a core sequence, additionally AU rich sequence 5'-thereof may be used to enhance the replicase activity from BMV. This oligo is to be used as the second primer. The first primer used with this second primer is a T7-based, HIV-specific primer.

T7 promoter

TCS = 87-34

20 TAATACGACTCACTATA|GGGA|CACCTAGGGCTAACTATGTGTCCTAATAAGG
(52 bases).

25 Additional, non-limiting details to aid in understanding the invention are provided in the examples that follow:

4. Examples

EXAMPLE I

10ml of blood from a patient suspected of being infected with a human immunodeficiency virus-type 1.

5 (HIV-1) is fractionated with a SepracellTM apparatus (Sepratech Corp., Oklahoma City, Oklahoma, U.S.A.) or, alternatively, with a Ficoll gradient, to isolate lymphocytes. The lymphocytes are then lysed and nucleic acid from them is isolated with an extractor apparatus
10 (Molecular Biosystems, Inc., San Diego, California, U.S.A.) or, alternatively, the lymphocytes are lysed by standard sodium dodecyl sulfate (SDS)-enzyme treatment and the nucleic acid isolated with reverse phase chromatography over DEAE cellulose. This is done as
15 follows:

Spin down cells: 5k rpm for 4' from 1 ml Tris-buffered saline (TBS), pH 7.5.

Draw off supernatant.

Resuspend pellet in:

20	500 μ l 0.3 M NaCl/20 mM Tris, pH 7.5	Mix well,
	100 μ l 2% SDS	then add
	200 μ l 5 mg/ml proteinase K	to
	200 μ l 0.25 M EDTA	pellet.

25 Vortex vigorously and incubate at 50°C for 45', vortexing for 10-15 seconds every 10'.

Load onto drained extractor column and allow to enter.

Wash column 1x 4 ml 0.3 M NaCl/20 mM Tris, pH 7.5.

Elute DNA/RNA with 4 ml 0.5 M NaCl/20 mM Tris pH 7.5.

30

To eluate add: 5 μ l glycogen
400 μ l 3 M NaOAc
9.0 ml ice-cold EtOH
mix well

- 5 Precipitate at -20°C overnight. A dry ice/ethanol bath for one hour may be used.
- Spin down DNA/RNA for 15' at 10,000 rpm in a swinging bucket rotor; pour off EtOH and drain dry on a kimwipe 2'.
- 10 Lyophilize for 10'.
- Resuspend pellet in 170 μ l TE.
- Incubate at 37°C for 5'.
- Pour a 1 ml spin column as follows:
- 15 Plug a 1 ml syringe with a small amount of glass wool (autoclaved).
Fill syringe with TE (tris-EDTA) treated with diethyl pyrocarbonate (DEPC).
Quickly add sephadex G-50 fine (in TE; autoclaved).
Continue to add until the solid matrix mounds over the top of the syringe.
- 20 Spin 30 seconds at 1000 rpm in an IEC table-top centrifuge.
Wash with 100 μ l TE, spin for 1' at medium speed (ca. 1000 rpm). Discard wash.
- 25 Load the extract onto the column. Spin again for 1'.
- Rinse with 150 μ l TE, spin at 1000 rpm for 1'. Pool rinse and the first fraction. Samples may be split for multiple reactions at this step.
- 30 Add 1/10 volume 8 M LiCl. Mix with 2.5 x vol. 100% EtOH. Vortex well. ppt. dry ice/EtOH for 30'.
- Spin down for 15' top speed in a microfuge at 4°C .
- Dry pellet.
- After the isolation, total nucleic acid from the sample is dissolved in 100 μ l of TE buffer (10mM Tris.Cl, 1mM EDTA, pH8). 10 μ l of the 100 μ l of total nucleic acid is dissolved to a final volume of 100 μ l containing:
- 40mM Tris.Cl, pH8
25mM NaCl

10mM MgCl₂
10mM dithiolthreitol (DTT)
2mM spermidine
100µg/ml bovine serum albumin
5 400mM each of dATP, dCTP, dGTP and TTP
30nM of the 101 base single-stranded DNA of
sequence 5'-GAACGCGGCT ACAATTAATA CATAACCTTA TGTATCATAC
ACATACGATT TAGGTGACAC TATA GAATAC TTTCGTAACA CTAGGCAAAG
GTGGCTTTAT C -3' primer A, is added. 30nM of the 29 base
10 DNA of sequence 5'-ACACCATATG TATGTTTCAG GGAAAGCTA-3',
primer B, is added. Primer B, which is the second primer,
has the sequence of bases 5151-5179 in the SOR gene of
the HIV-1. An alternative segment for the second primer,
corresponding to bases 5357-5387 of the SOR gene of the
15 HIV-1, has the sequence 5'-GCACACAAGT AGACCCTGAA
CTAGCAGACC A-3', and, if used, is also present at 30 nM.
Primer A is the first primer; its 64 5'-nucleotides form
the plus-strand of a promoter for the SP6 DNA-dependent
RNA polymerase. Its segment of sequence 5'-GAATAC-3' is
20 (alternative subsegment)₁, which includes the
transcription start site (the 5'-G) and other bases
apparently favored by the SP6 RNA polymerase at the 5'-end
of sequences transcribed by it. Finally, the 31
3'-terminal nucleotides form (primer subsegment)₁ and have
25 a sequence complementary to that of bases 5577-5547 in the
SOR gene of an HIV-1 isolate (See Ratner *et al.*, Nature
313, 277 (1985) for complete sequence.) (The HIV isolate
is referred to in these Examples as "the HIV-1")).

Note that all oligonucleotides employed in these
30 examples are made by solid-phase synthesis on an Applied
Biosystems, Inc. (Foster City, California, U.S.A.)
automated synthesizer (Model No. 380A) and are purified
chromatographically essentially to homogeneity by HPLC
using a C8 reverse phase column. Alternatively, other
35 commercially available synthesizers and standard

purification procedures could be used to prepare the oligonucleotides.

5 The 100 μ l of solution is heated at 65°C for 2 minutes and is then cooled to 42°C over the course of 1 minute. This heating and then holding at 42°C or above, in combination with the composition of the solution, provides conditions of stringency sufficient to provide hybridization of (3'-primer subsequent)₁, with sufficient stability to prime DNA synthesis, of high specificity to
10 the sequence complementary to that of (3'-primer subsequent)₁.

Then 10 units of avian myoblastosis virus (AMV) reverse transcriptase or 500 units of recombinant Moloney murine leukemia virus (MMLV) reverse transcriptase, as
15 purchased from Life Sciences, Inc., St. Petersburg, Florida, U.S.A., are added to the solution and the solution is incubated for 10 minutes at 42°C. (One unit incorporates 1nmole of TTP into acid-precipitable form in 10 minutes at 37°C using poly(A).oligo(T)₁₂₋₁₈ as
20 template-primer as defined by Houts *et al.*, J. Virol. 29, 517 (1979)). After the 10 minute incubation, the solution is placed in a boiling water bath for 1 minute. This heating causes strand-separation of the duplex formed by the reverse transcriptase.

25 Then the solution is cooled to 42°C over 1 minute. During this cooling, the second primer hybridizes to the 3'-terminal segment of the complement of target segment formed in the reaction catalyzed by the reverse transcriptase. Again, the hybridization conditions are
30 sufficiently stringent for sufficiently specific hybridization between the second primer and sequence complementary to that of second primer.

After the cooling, 10 additional units of AMV reverse transcriptase or 500 additional units of cloned
35 MMLV reverse transcriptase are added and further incubation for 10 minutes at 42°C is carried out.

Then RNasin^R brand ribonuclease inhibitor from Promega Biotec, Madison, Wisconsin, U.S.A. is added (optionally) to a concentration of 1 unit per ml. (1 unit is the amount of inhibitor required to inhibit by 50% the activity of 5ng of ribonuclease A. Roth, Meth. Cancer Res. 3, 151 (1976)). Further, each of the ribonucleoside 5'-triphosphates, ATP, GTP, CTP and UTP, is added to 400mM. Finally between 10 and 20 units of SP6 DNA-dependent RNA polymerase, purchased from Promega Biotec, are added and the resulting solution is incubated at 42°C for 30 to 60 minutes. (1 unit is the amount of the RNA polymerase required to catalyze the incorporation of 1 nmole of ribonucleoside triphosphate into acid insoluble product in 60 minutes at 37°C under standard reaction conditions (40mM Tris.Cl, pH 7.9, 6mM MgCl₂, 10mM DTT, 2mM spermidine, 0.5 mM of each of ATP, GTP, CTP and UTP, 0.5Ci of ³H-CTP, 1g of SP6 DNA and the enzyme in a total volume of 50μl.))

Then each of the following oligonucleotides is added to bring its concentration to 30nM:

third primer:

5'-GAACGCGGCT ACAATTAATA CATAACCTTA TGTATCATAC ACATACGATT
TAGGTGACAC TATA GAATAC ACTAATTCAT CTGTATTACT TTGACTGTTT
TTC-3'

fourth primer:

5'-TTTTTTGGTG TTATTAATGC TGCTAGTGCC-3'

In the third primer, as in the first, the 5'-terminal 64 bases are the promoter segment and the next 6 bases are the variable segment. The variable segment is the first six bases transcribed from the promoter by the SP6 RNA polymerase, and these bases are selected to enhance the level of such transcription. Finally, the (3'-primer subsegment)₃ portion of the third primer is the 3'-terminal 33 bases, in the same sequence as bases 5388-5420 in the short open reading frame (SOR) gene of the HIV-1. The fourth primer has the sequence

complementary to that of bases 5546-5517 of the SOR gene of the HIV-1.

After addition of the third and fourth primers, the solution is incubated at 42°C for 1 minute. During this period, hybridization occurs between (3'-primer subsegment)₃ of third primer and the first RNA formed in the reaction catalyzed by the SP6 RNA polymerase. Because of the stringency of the hybridization conditions, the (3'-primer subsegment)₃ hybridizes, with stability sufficient for priming of DNA synthesis, with high specificity to the segment of complementary sequence in the first RNA.

After the incubation, 10 units of AMV reverse transcriptase or 500 units of cloned MMLV reverse transcriptase are added and the solution incubated for 10 minutes at 42°C to form the third complementary DNA.

The solution is then suspended in a boiling water bath for 1 minute and cooled to 42°C over 1 minute to, first, render single-stranded the duplex between third complementary DNA and first RNA and, second, allow hybridization between fourth primer and third complementary DNA. As with the other hybridizations, the conditions are sufficiently stringent that hybridization of fourth primer, with stability sufficient to prime DNA synthesis, occurs with high specificity to the segment of third complementary DNA of sequence complementary to that of fourth primer.

Then, again, 10 units of AMV reverse transcriptase or 500 units of cloned MMLV reverse transcriptase are added and the solution is incubated for 10 minutes at 42°C.

Then RNasin^R brand ribonuclease inhibitor is added (optionally) to 1 unit per ml, followed by 10-20 units of SP6 RNA polymerase, and the solution is incubated for 30 minutes to 1 hour at 42°C.

The resulting second RNA can then be detected by a nucleic acid probe hybridization technique.

EXAMPLE II

The procedure of Example I is followed, with a modification noted below, with three samples: (A) one of 10ml of human blood known to be free of HIV, (B) one of 10ml of culture known to have about 10^3 HIV-1 infected cells per ml, and (C) one of 10ml of blood from a person suspected of being infected with an HIV-1. The modification of the procedure is that an alpha- ^{32}P -labeled ribonucleoside triphosphate is included as a substrate in the reaction catalyzed by the SP6 RNA polymerase to make the second RNA. The second RNA is, consequently, ^{32}P -labeled.

Sephacryl-S500TM macroporous beads are derivatized with a carboxyl-group-terminated linker (of formula- $\text{C}(=\text{NH})\text{NH}(\text{CH}_2)_5\text{CO}_2^-$) and then with 5'-(6-aminohexyl phosphoramidate)-derivatized oligonucleotide of sequence 5'-TGGTCTGCTA GTTCAGGGTC TACTTGTGTG C-3' which is the sequence complementary to that of bases 5357-5387 in the SOR gene of the HIV-1. (The sequence of bases 4901-4932 of the SOR gene occurs in any second RNA produced during amplification of nucleic acid from a sample.) Preparation of the beads was according to procedures described in commonly assigned United States Patent Application Serial No. 895,756, filed August 11, 1986, and incorporated herein by reference. Briefly, the support materials are porous silicate glass or macroporous cross-linked dextran, derivatized with an amino-terminated linker, with substantially all of the amino groups that are not covalently joined through a phosphoramidate group to the terminal nucleoside of the capture probe being blocked from interacting non-specifically with nucleic acid having an aliphatic acyl

group; macroporous cross-linked dextran activated with cyanogen bromide, which reacts with amines to link to aminoalkylphosphoramidate derivatized oligonucleotides; and porous silicate glass, macroporous cross-linked dextran or divinylbenzene-crosslinked polystyrene derivatized with a carboxyl-terminated or succinimide-ester-terminated linker, with a fraction of the carboxyl groups joined in an amide linkage to one amino group of a diaminoalkane, the other amino of which is part of a phosphoramidate which, in turn, is bonded directly to the terminal nucleoside of the capture probe. Preferred support materials are long chain alkylamine-derivatized and carboxyl-derivatized controlled pore glass as sold by Pierce Chemical Co., Rockford, Illinois, USA, under product numbers 24875 and 23735, respectively, in the form of beads with nominal pore diameter of 500 Angstroms and particle diameters between about 125 and 177 microns, and Sephacryl S-500, sold by Pharmacia Inc., Piscataway, New Jersey, USA under Code No. 17-0475-01 in the form of beads with a wet diameter of about 40 to about 105 microns and an exclusion volume for dextrans of molecular weight above about 2×10^7 daltons, said Sephacryl to be derivatized with cyanogen bromide or with an amino-terminated or carboxyl-terminated linker. In one aspect the solid support is one of:

(A) porous silicate glass derivatized at silicons with

(1) groups of formula

$-(CH_2)_n(NH)(CO)(CH_2)_cCH_3$ and

$-(CH_2)_n(NH)(PO_2)O-(Oligo),$

with substantially no silicon derivatized with a group terminated with an amino group, wherein c is 0 to 5, n is 2 to 8, $-O-(Oligo)$ is the oligonucleotide probe, and the oxygen atom bonded to $(Oligo)$ is the oligonucleotide probe, and the oxygen atom bonded to $(Oligo)$ is the 5'-

oxygen of the 5'-nucleoside or the 3'-oxygen of the 3'-nucleoside of the probe; or

(2) groups of formula

$-(CH_2)_n(NH)(CO)(CH_2)_mCO_2H$ and

5 $-(CH_2)_n(NH)(CO)(CH_2)_m(CO)(NH)(CH_2)_pNH(PO_2)O-(Oligo)$,
wherein m, n, and p are the same or different and are each
2 to 8; or

(B) a cross-linked dextran macroporous material
derivatized at hydroxyl oxygens, which are on carbons of
10 the sugar moieties which have at least one neighboring
carbon with an underivatized hydroxyl, with

(1) groups of formula

$-C(=NH)NH(CH_2)_q(NH)(CO)(CH_2)_cCH_3$ and

$-C(=NH)NH(CH_2)_p(NH)(PO_2)O-Oligo$,

15 with substantially no hydroxyl oxygen derivatized with a
group terminated with an amino group, or

(2) groups of formula

$-C(=NH)NH(CH_2)_qCO_2H$ and

$-C(=NH)NH(CH_2)_q(CO)(NH)(CH_2)_pNH(PO_2)O-(Oligo)$,

20 wherein c, q, and p are the same or different and q is 2
to 10; and

(C) divinylbenzene-crosslinked polystyrene
derivatized at phenyl groups with groups of formula

$-(CH_2)_sCO_2H$ and

25 $-(CH_2)_s(CO)(NH)(CH_2)_pNH(PO_2)O-(Oligo)$,

wherein s and p are the same or different and s is 2 to
10. See Ghosh et al., Nucleic Acids Research 15, 5353
(1987).

Each of three batches of 50mg of Sephacryl beads,
30 derivatized as described above, is soaked for 15 minutes
at 37°C C in 250 μ l of a prehybridization solution
containing 0.1% SDS, 10% dextran sulfate, 1mg/ml salmon
sperm DNA, and 5x SSPE (0.75M NaCl, 50mM NaH₂PO₄, pH 7.4,
and 5mM EDTA). After the soaking, the bead material is
35 pelleted by centrifugation and prehybridization solution
is removed by aspiration.

The nucleic acid from the amplification procedure on each of the three samples is isolated by ethanol precipitation and is then dissolved to 250 μ l in solution that is the same as pre-hybridization solution but lacks the salmon sperm DNA. Each of the resulting nucleic acid solutions is then combined with one batch of prehybridized oligonucleotide-derivatized Sephacryl beads and the resulting mixture is incubated with gentle agitation at 42°C C for 90 minutes.

The Sephacryl beads are then pelleted by centrifugation, hybridization solution is removed by aspiration, and the beads are then washed three times, 10 minutes each at 37°C C, in 1ml of a solution of 2 x SSC (0.30M NaCl, 0.03M Na.Citrate, pH 7.0). After each wash, the beads are pelleted by centrifugation and solution is removed by aspiration.

Immediately after the third wash, the beads are subjected to Cerenkov counting.

The beads with amplified nucleic acid from sample A produce a low level of counts, barely above background counts from scintillation fluid alone. The beads with amplified nucleic acid from sample B produce counts at a much higher level than that observed with beads associated with sample A. The beads with amplified nucleic acid from sample C, if they produce a level of counts significantly above the level produced with beads associated with sample A, indicate that the person, whose blood was taken for sample C, is infected with HIV-1.

EXAMPLE III

The procedures of Examples I and II are carried out with T7 RNA polymerase (Promega Biotec) in place of SP6 RNA polymerase, with each of the subsegment 5'-(promoter)₁-(variable subsegment)₁₋₃' of first primer and the subsegment 5'-(promoter)₃-(variable

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subsegment)₃-3' of third primer having the sequence 5'-TAATACGACT CACTATA GGGA-3', wherein the 17 5'-terminal bases are the promoter subsegment and the 4 3'-bases are the variable subsegment. The variable segment corresponds to the 4 5'-terminal bases of the transcript from the promoter. Ribonuclease inhibitor is not used in any step in this process, at least with the polymerase preparation from Promega Biotec.

EXAMPLE IV

The procedures of Examples I and II are carried out with T3 RNA polymerase (from Stratagene, San Diego, California) in place of the SP6 RNA polymerase and with the following segment used as the (promoter)₁-(variable subsegment)₁ subsegment of first primer and the (promoter)₃-(variable subsegment)₃ subsegment of third primer: 5'-TATTAACCCT CACTAAA GGGA-3', where the 17 5'-terminal bases are the promoter segment and the 4 3'-terminal bases the variable segment, including the 5'-terminal 4 bases in transcripts from the promoter.

EXAMPLE V

Employing the T7 RNA polymerase as in Example III, target segment in the genome of HIV from human blood samples is amplified using the following primers in place of the primers specified in Example I:

First Primer: 5'-TAATACGACT CACTATA GGGA TCTAATTACT ACCTCTTCTT CTGCTAGACT-3', wherein the 30 3'-terminal bases are complementary in sequence to bases 7076-7047 of the ENV gene of HIV-1.

Second Primer: 5'-ACAAGTTGTA ACACCTCAGT

40

CATTACACAG-3' with the sequence of bases 6838-6866 in the ENV gene of HIV-1.

Third Primer: Same 21 5'-terminal bases as first primer of this Example adjoined to the following 27
5 3'-terminal bases: 5'-AAAGGTATCC TTTGAGCCAA TTCCCATA-3',
which have the sequence of bases 6875-6902 in the ENV gene of the HIV-1.

Fourth Primer: 5'-AGTTGATACTACTGGCCTAATT-3', with the sequence complementary to that of bases 7033-7007 in
10 the ENV gene of HIV-1.

EXAMPLE VI

Employing the T7 RNA polymerase as in Example III, target segment in the genome of HIV from human blood samples is amplified using the following primers in place
15 of the primers specified in Example I:

First Primer: Same 21 5'-terminal nucleotides as the first and third primers of Example V adjoined to the following 31 3'-terminal nucleotides: 5'-CACCTAGGGC
20 TAACTATGTG TCCTAATAAG G-3', which have the sequence complementary to that of bases 5471-5441 of the SOR gene of HIV-1.

Second Primer: 5'-ACACCATATG TATGTTTCAG GGAAAGCTA-3', which has the same sequence as bases 5151-5179 of the SOR gene of HIV-1.

25 Third Primer: Same 21 5'-terminal nucleotides as the first and third primers of Example V adjoined to the following 31 3'-terminal nucleotides:
5'-AAGAATAAGTTCAGAAGTACACATCCCACT-3', which have the same sequence as bases 5220-5249 of the SOR gene of the HIV.

Fourth Primer: 5'-TGGTCTGCTAGTTCAGGGTCTACTTGTGTC-3', which has the sequence complementary to that of bases 5387-5357 in the SOR gene of HIV-1.

EXAMPLE VII

5 The procedure of Example I is followed for the isolation of nucleic acid from: 1) one sample containing 10^3 HIV-infected CEM cells mixed with 10^6 uninfected CEM cells (Cancer Center Research Foundation; CCRF-CEM; ATCC No. CCL 119); and 2) one sample containing 10^6 uninfected
10 CEM cells. These samples are resuspended in 100 μ l 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE) after the ethanol precipitation step to concentrate the sample obtained from the ExtractorTM column (MBI). The resuspended samples are fractionated on a Sephadex G-50 fine spin column
15 (Maniatis supra) eluting with TE. The eluted samples are concentrated by ethanol precipitation (in 0.8 M LiCl, 3 volumes ethanol, in dry ice/ethanol bath for 15 minutes). The precipitated sample is pelleted by centrifugation. The pellet is drained, dried, and then resuspended in 100
20 μ l containing 40 mM Tris-HCl, pH 8.1, 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine, 5 mM dithiothreitol, 100 μ M each dATP, dGTP, dCTP, and dTTP, 1 mM each rATP, rCTP, rGTP, and rUTP, 100 μ g/ml BSA (nuclease-free) and 250 nM each of DNA oligonucleotide primer A
25 (5'-AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTCCTAA-TAAGG-3') and primer B (5'-ACACCATATGTATGTTTCAGGGGAAAGCTA-3').

As controls, duplicate samples of purified HIV RNA at a concentration of 0.01 fm were resuspended in 100 μ l
30 of the buffer described above. Finally, a 10-fm β -globin sequence contained in plasmid H β 19A [Wallace et al., Nucl. Acids Res. 9, 3647 (1981), which had been linearized by HindIII, was resuspended in 100 μ l of the buffer described

above, except without the oligonucleotide primers. ,
Oligonucleotide primer D (5'-ACATTGCTTCTGACACAACTGTGTTCA-
')

and primer C (5'-TAATACGACTCACTATAGGGACAAAGGACTCAAA-3'),
5 which are specific for β -globin sequence, were added to
the β -globin reaction at 250 nM each.

Except for the β -globin sample, the reactions are
heated to 65°C for 1'. The β -globin sample is boiled for
1'. All samples are cooled to 42°C for 1 minute; then, 10
10 units of AMV reverse transcriptase are added. The
reactions are incubated for 15 minutes at 42°C, boiled for
1 minute, and then cooled at 42°C for 1 minute. Ten
additional units of AMV reverse transcriptase are added,
incubating at 42°C for 15 minutes. One hundred units of
15 T7 RNA polymerase are added to the reactions, and the
reactions are incubated at 37°C for 30 minutes.

The samples are boiled for 1 minute and cooled at
42°C for 1 minute, followed by the addition of 10 units
AMV reverse transcriptase. The reactions are incubated at
20 42°C for 15 minutes, boiled for 1 minute and cooled to
42°C for 1 minute. An additional 10 units of reverse
transcriptase are added, followed by incubation at 42°C
for 15 minutes. One hundred units of T7 RNA polymerase
are added, with a 30-minute incubation at 37°C. This
25 cycle is repeated two additional times.

The amplified target is then detected using
OligoBeads™ as described below.

Sephacryl beads containing HIV-1-specific
oligonucleotides were prepared as described and stored in
30 TE at 4°C at a concentration such that 250 μ l of the
suspension contains 50mg of Sephacryl beads.
Oligonucleotides were synthesized and HPLC purified as
described.

The oligonucleotides employed for both attachment
35 to the beads and detection are homologous to the SOR
region of the HIV-1 genome. The oligonucleotides used in

these studies were 86-31 (detection oligo nucleotide) (5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3') and 87-83 (capture oligo nucleotide on the bead:

5'-ATGCTAGATTGGTAATAACAACATATT-3'), which are homologous to the nonsense strand of the SOR region, and separated by approximately 100 nucleotides. For detection, the oligonucleotides were end labeled with 32 P according to a standard protocol. The unincorporated label was removed by gel filtration on a Sephadex G-50 fine column, and the oligonucleotide was stored at -20°C .

In a typical bead based sandwich hybridization system (BBSHS) experiment, the target and ^{32}P -labeled detection oligonucleotide are denatured in $10\mu\text{l}$ of TE containing 0.2% SDS at 65°C for 5 minutes in an Eppendorf tube. To this, $10\mu\text{l}$ of 2X Solution Hybridization Mix (10X SSPE/10% Dextran Sulfate) are added. The solution is mixed, centrifuged 2 seconds, and incubated at 42°C for 1 hour.

During this time the Sephacryl beads are pre-hybridized. The stock suspension of beads is mixed well, and $250\mu\text{l}$ aliquots (50 mg of beads) are transferred to Eppendorf tubes with mixing between each removal to ensure a uniform suspension. The aliquots are centrifuged for 10 seconds, and the TE is removed with a drawn-out Pasteur pipet. After $250\mu\text{l}$ of Hybridization Solution (5X SSPE [0.9M NaCl, 50 mM NaH_2PO_4 , pH 7.4, 1mM EDTA] 10% Dextran Sulfate/0.1% SDS) is added, the beads are suspended by gentle shaking and incubated 37°C for 30-60 minutes with occasional mixing. Immediately prior to the capture step, the beads are centrifuged for 10 seconds, the prehybridization solution is removed, $80\mu\text{l}$ of Hybridization Solution at 37°C are added, and the beads are returned to 37°C .

The solution hybridization is centrifuged for 2 seconds and transferred to the beads and Hybridization Solution. The beads are suspended and incubated at 37°C

for 1 hour with frequent mixing to maintain the suspension.

Following the capture, the beads are centrifuged 10 seconds and the Hybridization Solution, containing
 5 uncaptured target and detection oligonucleotide, is transferred to a scintillation counter vial. The beads are then washed 5 times with 2X SSC at 37°C. The first 3 washes are rapid; 1 ml of wash is added, the beads are mixed well and centrifuged 10 seconds, and the wash is
 10 transferred to a counting vial. For the final 2 washes, 1 ml of wash is added and the beads are mixed and incubated at 37°C for 5 minutes before being centrifuged. Each wash is counted separately to monitor the procedure.

Cerenkov counts of the Hybridization Solution, 5 washes, and beads are measured for 5-10 minutes. Counter background is subtracted from all samples. The fm target detected is calculated as follows:

(CPM on Beads/Total CPM) X fm Oligonucleotide
 Where total CPM is the sum of the CPM for the
 20 Hybridization Solution, 5 washes, and beads.

DETECTION OF AMPLIFIED TARGETS BY BBSHS

	TARGET	μ l USED	(FM) DETECTED 10 ⁻¹⁵ MOLES
25	10 ³ HIV infected cells;	0.33	0.06
	10 ⁶ CEM uninfected cells	0.66	0.13
	10 ⁶ uninfected CEM cell	0.07	0.01
		0.14	0.015
30		0.7	0.01
		10	0.008
	0.01 fm HIV RNA	0.007	0.14
		0.014	0.29
35	10 fm β -globin DNA	10	0.014

EXAMPLE VIII

The procedure of Example I is followed for isolation of nucleic acids from two samples: a) 10^3 HIV-infected CEM cells with 10^6 uninfected CEM cells; and b) 10^6 uninfected cultured CEM cells to which 0.01 fmoles of purified HIV are added after extraction. The procedure is then modified by further purification through a Sephadex G-50 (fine) spin column (Maniatis supra) eluting with TE after the Extractor column step. This is followed by an ethanol precipitation of the nucleic acid (in 0.8 M LiCl and 2.5 volume of EtOH). The nucleic acid is then resuspended in 100 μ l containing:

40 mM Tris-HCl, pH 8.1
8 mM $MgCl_2$
25 mM NaCl
2 mM spermidine
5 mM DTT (dithiothreitol)
100 μ M dATP, dTTP, dCTP, dGTP (each)
1 mM rATP, rCTP, rGTP, rUTP
100 μ g/ml BSA nuclease-free
250 nM 29 bp DNA oligonucleotide with the sequence
5'ACACCATATGTATGTTTCAGGGAAAGCTA-3' (Primer B)
250 nM 56 bp DNA oligonucleotide with the sequence
5'-
AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTGTCCTAA
TAAGG-3') (Primer A)

The 100 μ l of solution are heated to 65°C for 1 minute and cooled to 42°C over the course of 1 minute. This heating and then holding at 42°C or above, in combination with the composition of the solution, provides conditions of stringency sufficient for hybridization of (3'-primer subsegment)₁, (see Figure 1) with sufficient stability to the sequence complementary to that of (3'-primer subsegment)₁, in the target HIV RNA with high specificity.

Then, 10 units of avian myoblastosis virus (AMV) reverse transcriptase (Life Sciences, Inc.) are added to the solution, and the solution is incubated for 10 minutes at 42°C. [One unit incorporates 1 nmole of TTP into acid-precipitable form in 10 minutes at 37°C using poly(A)-oligo (T)12-18 as template-primer, as defined by Houts et al. J. Virol. 29, 517, (1979)]. After a 10 minute incubation, the solution is placed in a boiling water bath for one minute. This heating causes strand separation of the duplex formed by reverse transcriptase and inactivation of the reverse transcriptase.

Then, the solution is cooled to 42°C over one minute. During this cooling, the second primer, primer B, hybridizes to the 3'-terminal end (third subsegment)_{t2c}, see Figure 1, of the target complement of DNA strand formed in the reaction catalyzed by the reverse transcriptase in the previous step. Again, the hybridization conditions are sufficiently stringent for specific hybridization between the second primer and the sequence complementary to that of the second primer.

After cooling, 10 additional units of AMV reverse transcriptase are added and further incubation is carried out for 10 minutes at 42°C.

One hundred units of T7 polymerase are added to the reaction. The reaction is then incubated at 37°C for 30 minutes. An RNA transcript is synthesized which is complementary to the original target HIV RNA starting from the T7 promoter present at the 5' end of the duplex DNA template newly synthesized by the reverse transcriptase. The transcript has the sequence 5'-GGGA, then continues with sequence which is complementary to the second subsegment of the target sequence (i.e., second subsegment_{tcr}, see Figure 1). Since the reverse transcriptase has not been inactivated prior to this step, and since primer B is present, as well as the deoxynucleotide triphosphates, a secondary synthesis

occurs at this stage. Primer B hybridizes to the 3' region of the newly synthesized RNA transcript (third subsegment)t₂cr, see Figure 1, which is complementary to B primer. The reverse transcriptase (which had been added at the previous step) synthesizes a DNA strand using the newly synthesized RNA transcript (made by T7 polymerase) as a template, and using oligonucleotide B as the primer at the 5' end.

The reaction is then boiled for one minute to denature the RNA:DNA duplex. The reaction is then cooled to 42°C over one minute. During this cooling step, the target complementary segment of primer A hybridizes to the DNA strand synthesized during the previous step. At the same time, primer B hybridizes to its complementary sequence on the RNA transcript synthesized in the previous step.

After cooling, 10 additional units of AMV reverse transcriptase are added, and further incubation for 10 minutes at 42°C is carried out. Reverse transcriptase synthesizes DNA complementary to the HIV target using oligonucleotide A as a primer at the 5' end and DNA made in the previous step as a template. A second DNA strand is synthesized using oligonucleotide B as a primer and the RNA transcript made in the previous step as a template.

The reaction is boiled for one minute to denature the DNA duplex and the RNA:DNA duplex. The reaction is cooled to 42°C over one minute. Primer A hybridizes to the cDNA made using the RNA transcript as template in the previous step. (If the 3' end of the template strand is used as a reverse transcriptase primer also, a product identical to the end product of the next reaction is produced.) Primer B hybridizes to the DNA strand which is complementary to the target HIV RNA. This second synthesis produces a product which is a duplex DNA containing a T7 promoter sequence at its 5' end, as well as a 4 bp "variable" segment, 5'-GGGA-3'.

One hundred units of T7 polymerase are added to the reaction. The reaction is incubated for 30 minutes at 37°C. The T7 RNA polymerase uses the double-stranded duplex DNA containing a duplex T7 promoter as a template to transcribe an RNA which is complementary to the target second subsegment containing the additional sequence 5'-GGGA-3' at its 5' end.

This cycle (boil 1', 1' at 42°C, RT 10' at 42°C, RT 10' at 42°C, T7 polymerase 37°C 30') can be repeated as many times as needed to achieve the desired amplification of target sequence. The resulting product can then be detected by a nucleic acid probe hybridization technique.

EXAMPLE IX

Purified HIV RNA at a concentration of 1 fmole was resuspended in:

40 mM Tris-HCl, pH 8.1
8 mM MgCl₂
25 mM NaCl
2 mM spermidine
5 mM dithiothreitol
100 μM dATP, dTTP, dCTP, dGTP (each)
1 mM each rATP, rCTP, rGTP, rUTP
100 μg/ml BSA nuclease-free
250 nM 29 bp DNA oligonucleotide with the sequence
5'-ACACCATATGTATGTTTCAGGGAAAGCTA-3' (primer B)
250 nM 56 bp DNA oligonucleotide with the sequence
5-'AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTA
TGTGTCCTAATAAGG-3' (primer A)

The 100 μl of solution are heated to 65°C for 1 minute and cooled to 42°C over the course of 1 minute. This heating and cooling step, in combination with the composition of the solution, provides conditions of stringency sufficient for the hybridization of primer A with sufficient stability to the sequence complementary to

that of the primer A region in the target HIV RNA [(first subsegment) t_2 , in Figure 1] with high specificity.

Then, 10 units of avian myoblastosis virus (AMV) reverse transcriptase (Life Science, Inc.) are added to the solution, and the solution is incubated for 10 minutes at 42°C. After a ten-minute incubation, the solution is placed in a boiling water bath for one minute. This heating causes strand separation of the duplex formed by reverse transcriptase and inactivation of the reverse transcriptase.

The solution is cooled to 42°C over one minute. During this cooling, the second primer B hybridizes to the 3' end of the newly synthesized target-complementary strand [or (third subsegment) t_{2c} in Figure 1]. Again, the hybridization conditions are sufficiently stringent for specific hybridization between the second primer and the sequence complementary to that of the second primer.

After cooling, 10 additional units of AMV reverse transcriptase are added and further incubation is carried out for ten minutes at 42°C.

One hundred units of T7 RNA polymerase (New England Biolabs) are added to the reaction. The reaction is then incubated at 37°C for 30 minutes. An RNA transcript is synthesized which is complementary to the original target HIV RNA starting from the T7 promoter present at the 5' end of the duplex DNA template synthesized by the reverse transcriptase. The RNA transcript has the sequence 5'-GGGA, then continues with the sequence which is complementary to the second subsegment of the target sequence (i.e., second subsegment t_{cr} , Figure 1). Since the reverse transcriptase has not been inactivated prior to this step, and since primer B is present as well as the deoxynucleotide triphosphates, a secondary synthesis occurs at this stage. Primer B hybridizes to the 3' region of the newly synthesized RNA transcript [(third subsegment) t_{2cr} , see Figure 1] which is complementary to

primer B. The reverse transcriptase (which had been added at the previous step) synthesizes a DNA strand using the newly synthesized RNA transcript (made by the T7 RNA polymerase) as a template, and oligonucleotide B as a primer at the 5' end.

The reaction is then boiled for one minute to denature the RNA:DNA duplex. The reaction is then cooled to 42°C over one minute. During this cooling step, the target-complementary segment of primer A hybridizes to the DNA strand synthesized in the previous step. At the same time, primer B hybridizes to its complementary sequence on the RNA transcript synthesized in the previous step.

After cooling, 10 units of AMV reverse transcriptase are added, and further incubation for 10 minutes at 42°C is carried out. Reverse transcriptase synthesizes DNA complementary to the HIV target using oligonucleotide A as a primer at the 5' end and DNA made in the previous step as a template. The 3' end of the template strand is used as a primer to produce a final duplex DNA with a double-stranded, T7 promoter site at its 5' end. A second DNA strand is synthesized using oligonucleotide B as a primer and the RNA transcript made in the previous step as a template.

One hundred units of T7 polymerase are added to the reaction. The reaction is incubated for 30 minutes at 37°C. The T7 RNA polymerase synthesizes an RNA transcript using the duplex DNA containing the polymerase binding site (T7 promoter) at its 5' end as a template. The RNA transcript is complementary to the target second subsegment, see Figure 1, containing the additional sequence 5'-GGGA-3' at its 5' end. Further cycles may be performed to increase the amplification. The resulting products can be detected by a nucleic acid hybridization protocol.

EXAMPLE X

Protocol for Last-Round Labeling of a TAS Amplified Product

A. Column to remove unincorporated cold nucleotides

- 5 1. The TAS reaction (100 μ l) is taken after the cDNA synthesis in the final cycle of TAS. Add 400 μ l of Reagent A (0.1 M Tris-HCl, pH 7.7, 10 mM triethylamine, 1 mM disodium EDTA) to the reaction.
2. Equilibrate a NENSORB₂₀TM (Dupont) prepac
10 column by wetting the column material in 100% methanol (HPLC grade), then equilibrating with 2 ml Reagent A.
3. Load sample on column.
4. Wash the column 3 times with 1 ml Reagent A.
5. Wash the column 3 times with 1 ml water.
- 15 6. Elute the nucleic acids with 50% methanol, collecting 250-300 μ l fractions up to 1 ml total volume. (The first two fractions will contain the majority of the nucleic acid.)
7. Dry the fractions in the speed-vac or
20 lyophilizer.

B. Protocol to Label the Amplified Product

1. Resuspend the fractions from the NENSORB₂₀TM column (the first two fractions may be combined) in 40 mM Tris-HCl, pH 8.1, 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine-
25 (HCl)₃, 5 mM dithiothreitol, 400 μ M each rATP, rCTP, and rGTP, 12 μ M rUTP, and 25 μ Ci α -³²P-rUTP (800 Ci/mmol).
2. Add 50 units T7 RNA polymerase (New England Biolabs) for each 50 μ l of sample. Incubate at 37°C for 30 minutes.
- 30 3. The unincorporated label may be removed by a G-50 spin column (Maniatis supra).
4. The labeled sample may be run on a sequencing polyacrylamide gel to determine the size of the amplified

product. The labeled product may also be detected using the Oligo-BeadsTM.

EXAMPLE XI

Use of an Internal Standard During TAS Amplification

5 Samples were prepared having: 1) 0.1 fm HIV RNA and 0.1 fm β -globin DNA sequences (H β 19A cleaved with PstI); 2) 0.01 fm HIV RNA and 0.1 fm β -globin DNA (H β 19A cleaved with PstI); 3) 0.1 fm HIV RNA; or 4) 0.1 fm β -globin DNA (H β 19A cleaved with PstI in 100 μ l containing
10 40 mM Tris-HCl, pH 8.1, 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine-(HCl)₃, 5 mM dithiothreitol, 100 μ l each dATP, dTTP, dCTP and dGTP, 1 mM each rATP, rUTP, rCTP, and rGTP, 100 μ g/ml BSA (nuclease-free), and 250 nM primer A (5'-AATTTAATACGACTCACTATAGGGACACCTAGGGCTAACTATGTGTCCTAATAAGG-
15 3'), 250 nM primer B (5'-ACACCATATGTATGTTTCAGGGAAAGCTA-3'), 250 nM primer C (5'-TAATACGACTCACTATAGGGAAGCACTAAAGGCACCGAGCACTTTCTTGCC-3'), and 250 nM primer D (5'-ACATTGCTTCTGACACAAGTGTGTTCA-3'). A sample having 1/20th the starting nucleic acids was
20 placed into denaturing solution 7.4% formaldehyde, 10x SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.4) for the zero time-point samples.

 The samples were boiled for 1 minute and cooled at 42°C for 1 minute, followed by the addition of 10 units of
25 AMV reverse transcriptase. The reactions are incubated at 42°C for 15 minutes, boiled for 1 minute, and cooled at 42°C for 1 minute. An additional 10 units of AMV reverse transcriptase are added, followed by incubation at 42°C for 15 minutes. One hundred units of T7 RNA polymerase
30 are added, followed by incubation at 37°C for 30 minutes. This cycle is repeated a second time. (More cycles can be done, depending on the amplification needed.) Two samples containing 1/20th the starting target nucleic acids were placed into denaturing solution for the second
35 transcription time-point samples.

All samples were heated to 55°C for 30 minutes prior to filtering onto two nitrocellulose membranes. The nucleic acids were fixed to the membrane by irradiation with 254 nm UV light for 4 minutes. As controls, 1, 0.1, and 0.01 fm of plasmid pARV7/2 [Luciw *et al.*, *Nature* 312, 760 (1984)], which contains the entire HIV genome cDNA as well as plasmid H β 19A [Wallace *et al.*, *Nucl. Acids Res.* 9, 3647 (1981)], were denatured in 0.2 N NaOH, neutralized with an equal volume of 2 M ammonium acetate, and then filtered onto the nitrocellulose membrane. One membrane was hybridized with ³²p-labeled oligonucleotide 86-31¹, which is specific for the amplified product of HIV. The other membrane was hybridized to ³²p-labeled oligonucleotide 87-459², which will hybridize to the amplified β -globin product, as well as to the target β -globin plasmid.

The hybridizations were in 1% SDS, 0.9 M NaCl, 50 mM NaH₂PO₄ (pH 7.4), 5 mM EDTA (pH 7.4), and 10⁶ cpm/m. ³²p-labeled oligonucleotide for 1 hour at 55°C. The membranes were washed 3 times in 1% SDS, 0.18 M NaCl, 10 mM NaH₂PO₄ (pH 7.4), and 1 mM EDTA at room temperature, followed by 1 wash in the same buffer at 55°C.

The membranes were then autoradiographed for 16 hours on Kodak XAR film at -70°C with one intensifying screen.

Notes

1. 86-31: 5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3'
2. 87-459: 5'-AGGTTTAAGGAGACCAATAGAAACT-3'

The autoradiograph in Figure 3 shows the amount of HIV and β -globin sequences detected after two cycles of TAS carried out simultaneously on HIV and β -globin nucleic acid. The starting amount of β -globin was kept constant while the amount of HIV target was varied.

While the invention has been described with some specificity in the present specification, persons of ordinary skill in the pertinent arts will recognize variations and modifications of the invention as described that are within the spirit of the invention. Such variations and modifications are also within the scope of the invention as described and claimed herein.

WHAT IS CLAIMED IS:

1. A method of preparing a double-stranded nucleic acid containing a sequence corresponding to a target sequence operably linked to a promoter therefor, comprising:

providing a first nucleic acid primer containing a promoter sequence operably linked to a sequence corresponding to a segment of a target sequence,

hybridizing under suitable conditions said first nucleic acid primer with target sequence in a sample containing nucleic acid,

extending the hybridized said first nucleic acid primer in a polymerase extension reaction complementarily to the target sequence to form a corresponding duplex nucleic acid,

separating the strands of said duplex,

hybridizing to the separated promoter containing sequence strand under suitable conditions a second nucleic acid primer at the end opposite from said promoter sequence containing strand, and

extending the hybridized said second nucleic acid primer in a polymerase extension reaction complementarily to said promoter sequence containing strand.

2. The method according to Claim 1 repeated at least once.

3. A method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising employing the double-stranded nucleic acid according to Claim 1 as a double-stranded nucleic acid template for the preparation of a plurality of RNA transcripts therefrom, each bearing an RNA sequence corresponding to said target sequence, and detecting the presence of said RNA sequence.

4. A method of preparing a double-stranded nucleic acid containing a sequence corresponding to a

target sequence operably linked to a promoter therefor,
comprising providing a first nucleic acid primer
containing a promoter sequence operably linked to a
sequence complementary to a segment of a target sequence
5 and a second nucleic acid primer having a sequence
identical to a segment of a target sequence, said primers
corresponding to different regions of said target sequence
but not, or not substantially, overlapping in their
correspondence to the target and being selected such that
10 an extension product of one when separated from its
complement can serve as a template for an extension
product of the other, contacting a sample containing
nucleic acid including target sequence with said primers
under sequential hybridizing and strand separation
15 conditions so as to produce, in turn, extension products
of said primers.

5. A method comprising employing the double-
stranded nucleic acid according to Claim 1 or 4 as a
template for the preparation of a plurality of RNA
20 transcripts therefrom in a reaction catalyzed by a
polymerase that recognizes the promoter thereof, and
detecting the presence of said RNA transcripts.

6. The method according to Claim 3 or 5 wherein
said transcripts contain replicase recognition site for
25 replication of said transcripts by replicase induction.

7. The method according to Claim 3, 5 or 6
wherein the detected RNA sequence of said RNA transcripts
are measured in a standardized manner so as to measure the
amount of target sequence contained in a sample of nucleic
30 acid used in preparing the double-stranded nucleic acid
template.

8. The method according to Claim 7 wherein the
detected RNA sequence of said RNA transcripts is measured
in a manner internally standardized with the presence of a
35 known copy number of nucleic acid also contained in said
sample.

9. The method according to Claim 3 wherein said target sequence is disposed within a nucleic acid sequence associated with the characteristics of a genetic or pathogenic disease or condition.

5 10. The method according to Claim 9 wherein said nucleic acid sequence is a segment of a human immunodeficiency virus.

11. A method according to Claim 10 wherein said nucleic acid sequence is a segment of a defective gene.

10 12. A method according to Claim 5 wherein the promoter is a bacteriophage T7 promoter and the RNA transcripts are produced using T7 RNA polymerase.

13. A method according to Claim 5 wherein the promoter is a SP6 promoter and the RNA transcripts are produced using SP6 RNA polymerase.

15 14. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by E. coli DNA polymerase I.

20 15. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by Klenow fragment of E. coli DNA polymerase I.

16. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by T4 DNA polymerase.

25 17. A method according to Claim 1 or 4 wherein the extension reaction is catalyzed by reverse transcriptase.

18. A method according to Claim 3 or 5 wherein said RNA transcripts are labelled prior to detection.

19. A method according to Claim 18 wherein said RNA transcripts are radio-labelled.

30 20. A method according to Claim 18 wherein said RNA transcripts are chromophore labelled.

21. A kit useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising a first nucleic acid primer containing a promoter sequence operably linked to a sequence complementary to a segment of a target sequence

and a second nucleic acid primer having a sequence identical to a segment of a target sequence, said primers corresponding to different regions of said target sequence but not, or not substantially, overlapping in their
 5 correspondence to the target and being selected such that an extension product of one when separated from its complement can serve as a template for an extension product of the other, and means for hybridizing said first primer to said target sequence, chain extending the
 10 hybridized primer, strand-separating the resultant duplex, hybridizing to the promoter containing separated strand said second nucleic acid primer, chain extending the hybridized primer, causing the thus prepared double-stranded nucleic acid containing a promoter sequence to
 15 produce transcripts, and detecting said transcripts.

22. A method for amplifying a target nucleic acid segment of Formula I

3'-(first subsegment)_t-(second subsegment)_t-
 (third subsegment)_t-5'

20

I

wherein (first subsegment)_t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 3'-terminus of (second subsegment)_t,
 (second subsegment)_t is a nucleic acid segment of
 25 0 or more nucleotides, and (third subsegment)_t is a nucleic acid segment of known sequence of at least 10 nucleotides adjoining the 5'-terminus of (second subsegment)_t, which method comprises:

(1) hybridizing to (first subsegment)_t of said
 30 target segment a first primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula II

5'-(promoter)₁-(variable subsegment)₁-
 (3'-primer subsegment)₁-3'

59

II

wherein (promoter)₁ is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage DNA-dependent RNA polymerase-specific promoter, (variable subsegment)₁ is a single-stranded DNA segment of 0 to 100 nucleotides adjoining the 3'-terminal nucleotide of (promoter)₁ and the 5'-terminal nucleotide of (3'-primer subsegment)₁, and (3'-primer subsegment)₁ is a single-stranded DNA segment of at least 10 nucleotides with a sequence which is complementary to the sequence of a subsegment of (first subsegment)_t which terminates with the 3'-terminal nucleotide of (first subsegment)_t, said (promoter)₁ adjoining the 5'-terminus of said (3'-primer subsegment)₁, if said (variable subsegment)₁ has 0 nucleotides;

(2) extending said first primer, hybridized in accordance with step (1), in a reaction catalyzed by a first DNA polymerase to make a first complementary DNA segment, which comprises a subsegment of Formula III

5'-(promoter)₁-(variable subsegment)₁-(first subsegment)_{tC}-(second subsegment)_{tC}-(third subsegment)_{tC}-3',

III

wherein (first subsegment)_{tC} is the DNA segment with the sequence complementary to that of (first subsegment)_t, (second subsegment)_{tC} is the DNA segment with the sequence complementary to that of (second subsegment)_t, and (third subsegment)_{tC} is the DNA segment with the sequence complementary to that of (third subsegment)_t, provided that, if the target segment is an RNA segment, said first DNA polymerase is a reverse transcriptase;

60

(3) rendering single-stranded the duplex formed in the reaction of step (2);

(4) hybridizing to (third subsegment)_{tc} of said first complementary DNA of Formula III a second primer, which is a single-stranded DNA of at least 10 nucleotides of Formula IV

3'-(5'-primer subsegment)₂-(variable subsegment)₂-5',

IV

wherein (5'-primer subsegment)₂ has the sequence of a subsegment of (third subsegment)_t which terminates with the 5'-terminal nucleotide of (third subsegment)_t and wherein (variable subsegment)₂ is a segment of 0 to 100 nucleotides which adjoins the 5'-terminus of (5'-primer subsegment)₂;

(5) extending said second primer segment, hybridized in accordance with step (4), in a reaction catalyzed by a second DNA polymerase to form a second complementary DNA segment which comprises a subsegment of Formula V

5'-(variable subsegment)₂-(third subsegment)_t-(second subsegment)_t-(first subsegment)_t-(variable subsegment)_{1c}-(promoter)_{1c}-3',

V

wherein (variable subsegment)_{1c} is the DNA segment with the sequence complementary to that of (variable subsegment)₁, and (promoter)_{1c} is the DNA segment with the sequence complementary to that of (promoter)₁, provided that said second DNA polymerase is the same as or different from said first DNA polymerase; and

(6) employing the double-stranded product of step (5) as the template in a reaction catalyzed by a first

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bacteriophage DNA-dependent RNA polymerase, which recognizes the promoter of which one strand is (promoter-)₁, to make a first RNA product of Formula VI

5'- (variable subsegment)_{1r}- (first subsegment)_{tcr}-
 5 (second subsegment)_{tcr}- (third subsegment)_{tcr}-
 (variable subsegment)_{2cr}-3'

VI

wherein (variable subsegment)_{1r} is the RNA segment with the sequence of (variable subsegment)₁, (first subsegment)_{tcr} is the RNA segment with the sequence complementary to that of (first subsegment)_t, (second subsegment)_{tcr} is the RNA segment with the sequence complementary to that of
 10 (second subsegment)_t, (third subsegment)_{tcr} is the RNA segment with the sequence complementary to that of (third subsegment)_t, and (variable subsegment)_{2cr} is the RNA segment with the sequence complementary to that of
 15 (variable subsegment)₂.

23. A method according to Claim 22 wherein
 20 (first subsegment)_t is at least 10 nucleotides in length and is of Formula XIII

3'- (first subsegment)_{t2}- (first subsegment)_{t1}-5',

XIII

wherein (first subsegment)_{t2} has 0 or more nucleotides
 25 and, if more than 0, adjoins the 3'-terminus of (first subsegment)_{t1}, and (first subsegment)_{t1} is at least 10 nucleotides in length; wherein (third subsegment)_t is of Formula XIV

3'- (third subsegment)_{t1}- (third subsegment)_{t2}-5',

30

XIV

wherein (third subsegment)_{t2} has 0 or more nucleotides and, if more than 0, adjoins the 5'-terminus of (third subsegment)_{t1}, and (third subsegment)_{t1} is at least 10

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nucleotides in length; wherein (3'-primer subsegment)₁ has the sequence complementary to that of a subsegment of target segment which consists of all of (first subsegment)_{t2} and 0 or more nucleotides of (first subsegment)_{t1}; wherein (5'-primer subsegment)₂ is a subsegment which consists of all of (third subsegment)_{t2} and 0 or more nucleotides of (third subsegment)_{t1}; wherein (variable subsegment)₂ has 0 nucleotides; and wherein, after steps (1) to (6) of Claim 1, the RNA subsegment of Formula VII

5'-(first subsegment)_{t1cr}-(second subsegment)_{tcr}-(third subsegment)_{t1cr}-3'

VII

wherein (first subsegment)_{t1cr} is the RNA segment with the sequence complementary to that of (first subsegment)_{t1} and (third subsegment)_{t1cr} is the RNA segment with the sequence complementary to that of (third subsegment)_{t1}, is further amplified by a method which comprises:

(7) hybridizing to said first RNA product of Formula VI a third primer, which is a single-stranded DNA which comprises a 3'-terminal subsegment of Formula VIII

5'-(promoter)₃-(variable subsegment)₃-(3'-primer subsegment)₃-3',

VIII

wherein (promoter)₃ is a single-stranded DNA segment with the sequence of the plus-strand of a bacteriophage DNA-dependent RNA polymerase-specific promoter, said sequence of (promoter)₃ being the same as or different from that of (promoter)₁, (variable subsegment)₃ is a single-stranded DNA segment of 0 to 100 nucleotides which adjoins the 3'-terminal nucleotide of (promoter)₃ and the 5'-terminal nucleotide

of (3'-primer subsegment)₃, and (3'-primer subsegment)₃ is a single-stranded DNA segment which has the same sequence as

(third subsegment)_{t1} and adjoins the 3'-terminal nucleotide of (promoter)₃ if (variable subsegment)₃ has 0 nucleotides;

(8) extending said third primer hybridized in accordance with step (7) in a reaction catalyzed by a third DNA polymerase, which is a reverse transcriptase and is the same as or different from said first and second DNA polymerase, to make a third complementary DNA segment which comprises a 3'-terminal subsegment of Formula IX

5'-(promoter)₃-(variable subsegment)₃-(third subsegment)_{t1}-(second subsegment)_t-(first subsegment)_{t1}-(first subsegment)_{t2}-(variable subsegment)_{1c}-3',

IX

wherein (variable subsegment)_{1c} is the DNA with the sequence complementary to that of (variable subsegment)₁;

(9) rendering single-stranded the duplex formed in the reaction of step (8);

(10) hybridizing to the third complementary DNA made in the reaction of step (8) a fourth primer of Formula X

5'-(variable subsegment)₄-(5'-primer subsegment)₄^{-3'}

X

wherein (variable subsegment)₄ is a segment of 0 to 100 nucleotides, and (5'-primer subsegment)₄ is a subsegment of known sequence which adjoins the 3'-nucleotide of (variable subsegment)₄, if (variable subsegment)₄ has more

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than 0 nucleotides, and which comprises at least 10 nucleotides of the segment of Formula XX

5'-(variable subsegment)₁-(first subsegment)_{t2c}-
(first subsegment)_{t1c}-3',

5

XX

wherein (first subsegment)_{t2c} is the DNA segment with the sequence complementary to that of

(first subsegment)_{t2} and (first subsegment)_{t1c} is the DNA segment with the sequence complementary to that of (first subsegment)_{t1}, provided that at least one of said at least 10 nucleotides is at or 5' from the 5'-terminal nucleotide of (first subsegment)_{t1c};

(11) extending said fourth primer hybridized in accordance with step (10) in a reaction catalyzed by a fourth DNA polymerase, which is the same as or different from said first, second and third DNA polymerase, to form a fourth complementary DNA which comprises a segment of Formula XI

5'-(first subsegment)_{t1c}-(second segment)_{tc}-
(third subsegment)_{t1c}-(variable subsegment)_{3c}-
(promoter)_{3c}-3',

20

XI

wherein (second subsegment)_{tc} is the DNA segment with the sequence complementary to that of

(second subsegment)_t, (third subsegment)_{t1c} is the DNA segment with the sequence complementary to that of (third subsegment)_{t1},

(variable subsegment)_{3c} is the DNA segment with the sequence complementary to that of (variable segment)₃, and (promoter)_{3c} is the DNA segment with the sequence complementary to that of (promoter)₃; and

30

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(12) employing the double-stranded product of step (11) as the template in a reaction catalyzed by a second bacteriophage DNA-dependent RNA polymerase, which is the same as or different from said first bacteriophage DNA-dependent RNA polymerase and which recognizes the promoter of which one strand is (promoter)₃, to make a second RNA product with a 5'-terminal subsegment of Formula XII

5'- (variable subsegment)_{3r}- (third subsegment)_{t1r}-
 (second subsegment)_{tr}- (first subsegment)_{t1r}-
 X₁₂- (variable subsegment)_{4cr}-3',

XII

wherein (variable subsegment)_{3r} is the RNA segment with the sequence of (variable subsegment)₃,

(third subsegment)_{t1r} is the RNA segment with the sequence of (third subsegment)_{t1},

(second subsegment)_{tr} is the RNA segment with the sequence of (second subsegment)_t,

(first subsegment)_{t1r} is the RNA segment with the sequence of (first subsegment)_{t1},

(variable subsegment)_{4cr} is the RNA segment with the sequence complementary to that of (variable subsegment)₄, and X₁₂ is the RNA segment with the sequence complementary to that of the subsegment of

(5'-primer subsegment)₄ that is 5' from the 5'-terminus of (first subsegment)_{t1c}.

24. A method according to Claim 22 wherein each of (variable subsegment)₁ and (variable subsegment)₂ has 0 to 60 nucleotides, each of (3'-primer subsegment)₁ and (5'-primer subsegment)₂ has 15 to 45 nucleotides, (second subsegment)_t has at least 30 nucleotides, and target segment has 1000 or fewer nucleotides.

25. A method according to Claim 23 wherein the target segment is a DNA segment, wherein the product of step(2) is rendered single-stranded by thermal

denaturation, wherein (variable subsegment)₂ has 0 nucleotides, wherein each of the first and second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, Thermus aquaticus heat-stable DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase, and wherein the bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase.

26. A method according to Claim 25 wherein each of the first and second DNA polymerase is selected from the group consisting of Klenow Fragment of E. coli DNA polymerase I, AMV reverse transcriptase, and cloned MMLV reverse transcriptase.

27. A method according to Claim 26 wherein (1) the bacteriophage DNA-dependent RNA polymerase is T7 RNA polymerase, (promoter)₁ is 5'-TAATACGACTCACTATA-3' and (variable subsegment)₁ has a dinucleotide of sequence 5'-GG-3' at its 5'-terminus; or (2) the bacteriophage DNA-dependent RNA polymerase is T3 RNA polymerase, (promoter)₁ is 5'-TATTAACCCTCACTAAA-3' and (variable subsegment)₁ has a tetranucleotide of sequence 5'-GGGA-3' at its 5'-terminus; or (3) the bacteriophage DNA-dependent RNA polymerase is SP6 RNA polymerase, (promoter)₁ is 5'-GAACGCGGCTACAATTAATACATAACCTTATGTATCATAACATACGATTTAGGTGACACTATA-3', and (variable subsegment)₁ has a hexanucleotide of sequence 5'-GAATAC-3' at its 5'-terminus.

28. A method according to Claim 27 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)₁.

29. A method according to Claim 28 wherein, if the bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment)₁ has the sequence 5'-GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3' and, if the

bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)₁ has the sequence 5'-GAATACTGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3'.

30. A method according to Claim 24 wherein the
5 target segment is a RNA segment, wherein the product of
step(2) is rendered single-stranded by thermal denatura-
tion, wherein (variable subsegment)₂ has 0 nucleotides,
wherein the first DNA polymerase is selected from the
group consisting of AMV reverse transcriptase and cloned
10 MMLV reverse transcriptase, wherein the second DNA
polymerase is selected from the group consisting of Klenow
Fragment of E. coli DNA polymerase I, AMV reverse
transcriptase, cloned MMLV reverse transcriptase, calf
thymus DNA polymerase alpha, Thermus aquaticus
15 heat-stable DNA polymerase, and SequenaseTM brand cloned
T7 DNA polymerase, and wherein the bacteriophage
DNA-dependent RNA polymerase is selected from the group
consisting of T7 RNA polymerase, T3 RNA polymerase and SP6
RNA polymerase.

20 31. A method according to Claim 30 wherein the
second DNA polymerase is selected from the group
consisting of Klenow Fragment of E. coli DNA polymerase I,
AMV reverse transcriptase, and cloned MMLV reverse
transcriptase.

25 32. A method according to Claim 31 wherein (1) the
bacteriophage DNA-dependent RNA polymerase is T7 RNA
polymerase, (promoter)₁ is 5'-TAATACGACTCACTATA-3' and
(variable subsegment)₁ has a dinucleotide of sequence
5'-GG-3' at its 5'-terminus; or (2) the bacteriophage
30 DNA-dependent RNA polymerase is T3 RNA polymerase,
(promoter)₁ is 5'-TATTAACCCTCACTAAA-3' and (variable
subsegment)₁ has a tetranucleotide of sequence
5'-GGGA-3' at its 5'-terminus; or (3) the bacteriophage
DNA-dependent RNA polymerase is SP6 RNA polymerase,
35 (promoter)₁ is 5'-GAACGCGGCTACAATTAATACATAAC-
CTTATGTATCATACATACGATTAGGTGACACTATA-3', and (variable

subsegment)₁ has a hexanucleotide of sequence 5'-GAATAC-3' at its 5'-terminus.

33. A method according to Claim 32 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)₁.

34. A method according to Claim 33 wherein each of the first and second DNA polymerase is selected from AMV reverse transcriptase and cloned MMLV reverse transcriptase.

35. A method according to Claim 34 wherein, if the bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment)₁ has the sequence 5'-GGGATGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3' and, if the bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)₁ has the sequence 5'-GAATACTGGGGAACCCCCCTTCGGGGGTCACCTCGCGCAGC-3'.

36. A method according to Claim 30, 31, 32, 33, 34 or 35 wherein the target segment is a segment of the genome of a human immunodeficiency virus that is an HIV-1 virus and wherein (1) (3'-primer subsegment)₁ has the sequence 5'-TCTAATTACTACCTCTTCTTCTGCTAGACT-3' and (5'-primer CCTCTTCTTCTGCTAGACT-3' and (5'-primer subsegment)₂ has the sequence 5'-ACAAGTTGTAACACCTCAGTCATTACACAG-3' or (2) (3'-primer subsegment)₁ has the sequence 5'-TTTCGTAACACTAGGCAAAGGTGGCTTTATC-3' and (5'-primer subsegment)₂ has a sequence selected from the group consisting of 5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3' and 5'-ACACCATATGTATGTTTCAGGGAAAGCTA-3'.

37. A method according to Claim 23 wherein each of (variable subsegment)₁ and (variable subsegment)₃ has 0 to 60 nucleotides, wherein each of (3'-primer subsegment)₁, (5'-primer subsegment)₂, (3'-primer subsegment)₃, and (5'-primer subsegment)₄ has 15 to 45 nucleotides, (second subsegment)_t has at least 30 nucleotides, and target segment has 1000 or fewer nucleotides.

38. A method according to Claim 37 wherein (A) if the target segment is a DNA segment, the product of step (2) is rendered single-stranded by thermal denaturation and the product of step (8) is rendered single-stranded by thermal denaturation; each of the first, second and fourth DNA polymerase is selected from the group consisting of Klenow Fragment of *E. coli* DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, *Thermus aquaticus* heat-stable DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase; the third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase; and the each of the first and second bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase; and (B) if the target segment is a RNA segment, each of the product of step (2) and the product of step (8) is rendered single-stranded by thermal denaturation; each of the second and fourth DNA polymerase is selected from the group consisting of Klenow Fragment of *E. coli* DNA polymerase I, AMV reverse transcriptase, cloned MMLV reverse transcriptase, calf thymus DNA polymerase alpha, *Thermus aquaticus* heat-stable DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase; each of the first and third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase; and the each of the first and second bacteriophage DNA-dependent RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase.

39. A method according to Claim 38 wherein the target segment is a RNA segment and wherein each of the second and fourth DNA polymerase is selected from the group consisting of Klenow Fragment of *E. coli* DNA polymerase I, AMV reverse transcriptase , and cloned MMLV

reverse transcriptase and wherein each of the first and third DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase.

5 40. A method according to Claim 38 wherein the subsegment of target segment which has the sequence complementary to that of (3'-primer subsegment)₁ is in the 3'-direction from and does not overlap the subsegment of target segment which has the sequence complementary to
10 that of (5'-primer subsegment)₄, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)₂ does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)₃.

15 41. A method according to Claim 40 wherein the subsegment of third complementary DNA with the sequence complementary to that of (3'-primer subsegment)₁ is in the 5'-direction from and does not overlap the subsegment of third complementary DNA which has the sequence
20 complementary to that of (5'-primer subsegment)₄, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)₂ does not overlap the subsegment of target segment which has the sequence of (3'-primer
25 subsegment)₃.

 42. A method according to Claim 41 wherein, in the third complementary DNA, (variable subsegment)_{1c} has more than 0 nucleotides and wherein, in the duplex between (5'-primer subsegment)₄ and third complementary DNA, at
30 least a subsegment of (5'-primer subsegment)₄ is hybridized to (variable subsegment)_{1c}.

 43. A method according to Claim 41 wherein the subsegment of target segment which has the sequence complementary to that of (3'-primer subsegment)₁ is in the
35 3'-direction from and does not overlap the subsegment of target segment which has the sequence complementary to

that of (5'-primer subsegment)₄, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)₂ does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)₃.

44. A method according to Claim 41 wherein the subsegment of third complementary DNA with the sequence complementary to that of (3'-primer subsegment)₁ is in the 5'-direction from and does not overlap the subsegment of third complementary DNA which has the sequence complementary to that of (5'-primer subsegment)₄, and wherein the subsegment of target segment which has the sequence of (5'-primer subsegment)₂ does not overlap the subsegment of target segment which has the sequence of (3'-primer subsegment)₃.

45. A method according to Claim 44 wherein, in the third complementary DNA, (variable subsegment)_{1c} has more than 0 nucleotides and wherein, in the duplex between (5'-primer subsegment)₄ and third complementary DNA, at least a subsegment of (5'-primer subsegment)₄ is hybridized to (variable subsegment)_{1c}.

46. A method according to Claim 40, 41, 42, 43, 44 or 45 wherein (1) if T7 RNA polymerase is employed in step (6) or step (12), (promoter)₁, if the step is step (6), or (promoter)₃, if the step is step (12), has the sequence 5'-TAATACGACTCACTATA-3' and (variable subsegment)₁, if the step is step (6), or (variable subsegment)₃, if the step is step (12), has a dinucleotide of sequence 5'-GG-3' at its 5'-terminus; or (2) if T3 RNA polymerase is employed in step (6) or step (12), (promoter)₁, if the step is step (6), or (promoter)₃, if the step is step (12), has the sequence 5'-TATTAACCCTCACTAAA-3' and (variable subsegment)₁, if the step is step (6), or (variable subsegment)₃, if the step is step (12), has a tetranucleotide of sequence 5'-GGGA-3' at its 5'-terminus; or (3) if the SP6 RNA polymerase is employed in step (6)

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or step (12), (promoter)₁, if the step is step (6), or (promoter)₃, if the step is step (12), has the sequence 5'-GAACGCGGCTACAATTAATACATAACCTTATGTATCATAACATACGAT-TTAGGTGACACTATA-3', and (variable subsegment)₁, if the
 5 step is step (6), or (variable subsegment)₃, if the step is step (12) has a hexanucleotide of sequence 5'-GAATAC-3' at its 5'-terminus; and wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)₁ and the 5'-terminus of the third primer is the 5'-nucleotide of
 10 (promoter)₃.

47. A method according to Claim 40, 41, 42, 43, 44 or 45 wherein (A)(i) if the first bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment)₁ has the sequence
 15 5'-GGGATGGGGAACCCCCCTTCGGGGGTACCTCGCGCAGC-3' and second primer (variable subsegment)₂ has the sequence 5'-CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3'; or (ii) if the first bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)₁ has the sequence 5'-GAATACTGGGGAACCCCCCTTCGG-
 20 GGGTCACCTCGCGCAGC-3' and second primer (variable subsegment)₂ has the sequence 5'-CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3' (B)(i) if the second bacteriophage DNA-dependent RNA polymerase is the T7 or T3 RNA polymerase, (variable subsegment)₃ has the sequence
 25 5'-GGGATGGGGAACCCCCCTTCGGGGGTACCTCGCGCAGC-3'; (ii) if the second bacteriophage DNA-dependent RNA polymerase is the SP6 RNA polymerase, (variable subsegment)₃ has the sequence 5'-GAATACGGGATGGGGAACCCCCCTTCGGGGGTCACTCGCGCAGC-
 30 3' and the fourth primer has as the 5'terminal 40 nucleotides 5'-CGCGCTCTCCCAGGTGACGCCTCGAGAAGAGGCGCGACCTTCGTGC-3'; and (C) after step (12), the second RNA is autocatalytically
 35 replicated with Q β replicase.

48. A method according to Claim 37, 38, 39, 40, 41, 42, 43, 44 or 45 wherein the target segment is a segment of the genome of a human immunodeficiency virus that is an HIV-1 virus and wherein (1) (3'-primer subsegment)₁ has
5 the sequence 5'-TCTAATTACTACCTCTTCTTCTGCTAGACT-3',
(5'-primer subsegment)₂ has the sequence
5'-ACAAGTTGTAACACCTCAGTCATTACACAG-3',
(3'-primer subsegment)₃ has the sequence
5'-AAAGGTATCCTTTGAGCCAATTCCCATA-3', and fourth primer has
10 the sequence 5'-AGTTGATACTACTGGCCTAATT-3'; or (2)
(3'-primer subsegment)₁ has the sequence
5'-TTTCGTAACACTAGGCAAAGGTGGCTTTATC-3', (5'-primer
subsegment)₂ has a sequence selected from the group
consisting of 5'-GCACACAAGTAGACCCTGAACTAGCAGACCA-3' and
15 5'-ACACCATATGTATGTTTCAGGGAAAGCTA-3',
(3'-primer subsegment)₃ has the sequence
5'-ACTAATTCATCTGTATTACTTTGACTGTTTTTC-3', and fourth primer
has the sequence 5'-TTTTTTGGTGTATTAATGCTGCTAGTGCC-3'.

49. A method according to Claim 24 wherein (A) if
20 the target segment is a DNA segment, the product of step
(2) is rendered single-stranded by thermal denaturation;
each of the first and second DNA polymerase is selected
from the group consisting of Klenow Fragment of E. coli
DNA polymerase I, AMV reverse transcriptase, cloned MMLV
25 reverse transcriptase, calf thymus DNA polymerase alpha,
Thermus aquaticus heat-stable DNA polymerase, and
SequenaseTM brand cloned T7 DNA polymerase; and the first
bacteriophage DNA-dependent RNA polymerase is selected
from the group consisting of T7 RNA polymerase, T3 RNA
30 polymerase and SP6 RNA polymerase; and (B) if the target
segment is a RNA segment, the product of step (2) is
rendered single-stranded by thermal denaturation; the
second DNA polymerase is selected from the group
consisting of Klenow Fragment of E. coli DNA polymerase I,
35 AMV reverse transcriptase, cloned MMLV reverse
transcriptase, calf thymus DNA polymerase alpha, Thermus

aquaticus heat-stable DNA polymerase, and SequenaseTM brand cloned T7 DNA polymerase; the first DNA polymerase is selected from the group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase; and
5 the first bacteriophage DNA-dependent RNA polymerase is selected from the T7 RNA polymerase, the T3 RNA polymerase and the SP6 RNA polymerase.

50. A method according to Claim 49 wherein each of the first and second DNA polymerase is selected from the
10 group consisting of AMV reverse transcriptase and cloned MMLV reverse transcriptase.

51. A method according to Claim 50 wherein the 5'-terminus of the first primer is the 5'-nucleotide of (promoter)₁; wherein, if the bacteriophage DNA-dependent
15 RNA polymerase is the T7 RNA polymerase is the T7 RNA polymerase, the subsegment (promoter)₁-(variable subsegment)₁ of the first primer has the sequence 5'-TAATACGACTCACTATAGGGACGCGCTCTCCCAGGTGACGCCTC-GAGAAGAGGCGCGACCTTCGTGC-3'; wherein, if the bacteriophage
20 DNA-dependent RNA polymerase is the T3 RNA polymerase, the subsegment (promoter)₁-(variable subsegment)₁ of the first primer has the sequence 5'-TATTAACCCTCACTAAAGGGACGCGCTCTCCCAGGTGACGCCTC-GAGAAGAGGCGCGACCTTCGTGC-3'; wherein, if the bacteriophage
25 DNA-dependent RNA polymerase is the SP6 RNA polymerase, the subsegment (promoter)₁-(variable subsegment)₁ of the first primer has the sequence 5'-GAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGAT-TTAGGTGACACTATAGAATACCGCGCTCTCCCAGGTGACGCCTCGAGAAGAG-GCGCGACCTTCGTGC-3'; wherein (variable subsegment)₂ has the
30 sequence 5'-TGGGGAACCCCTTCGGGGGTACCTCGCGCAGC-3'; and wherein, after step (6), the first RNA is autocatalytically replicated with Q β replicase.

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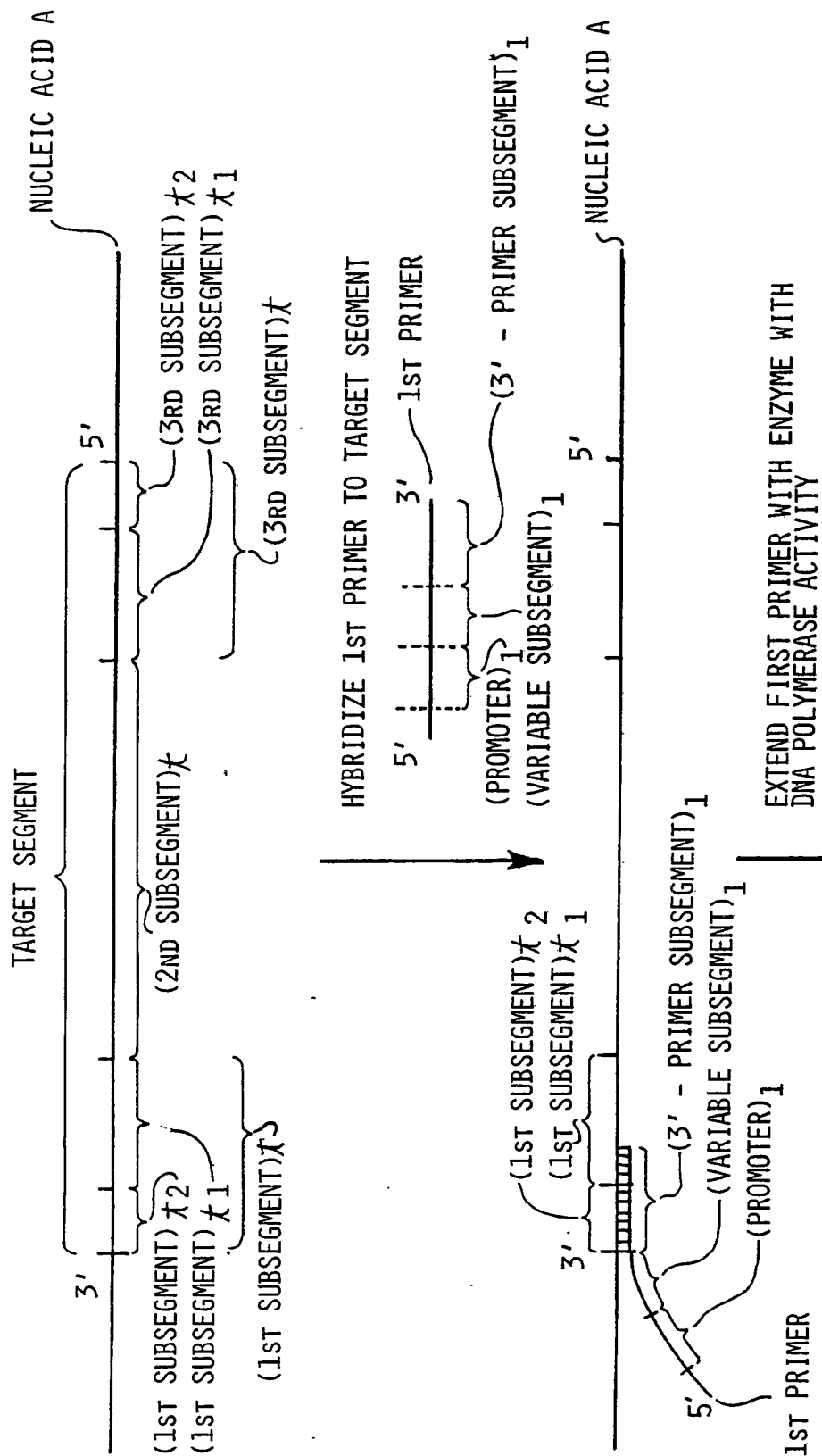
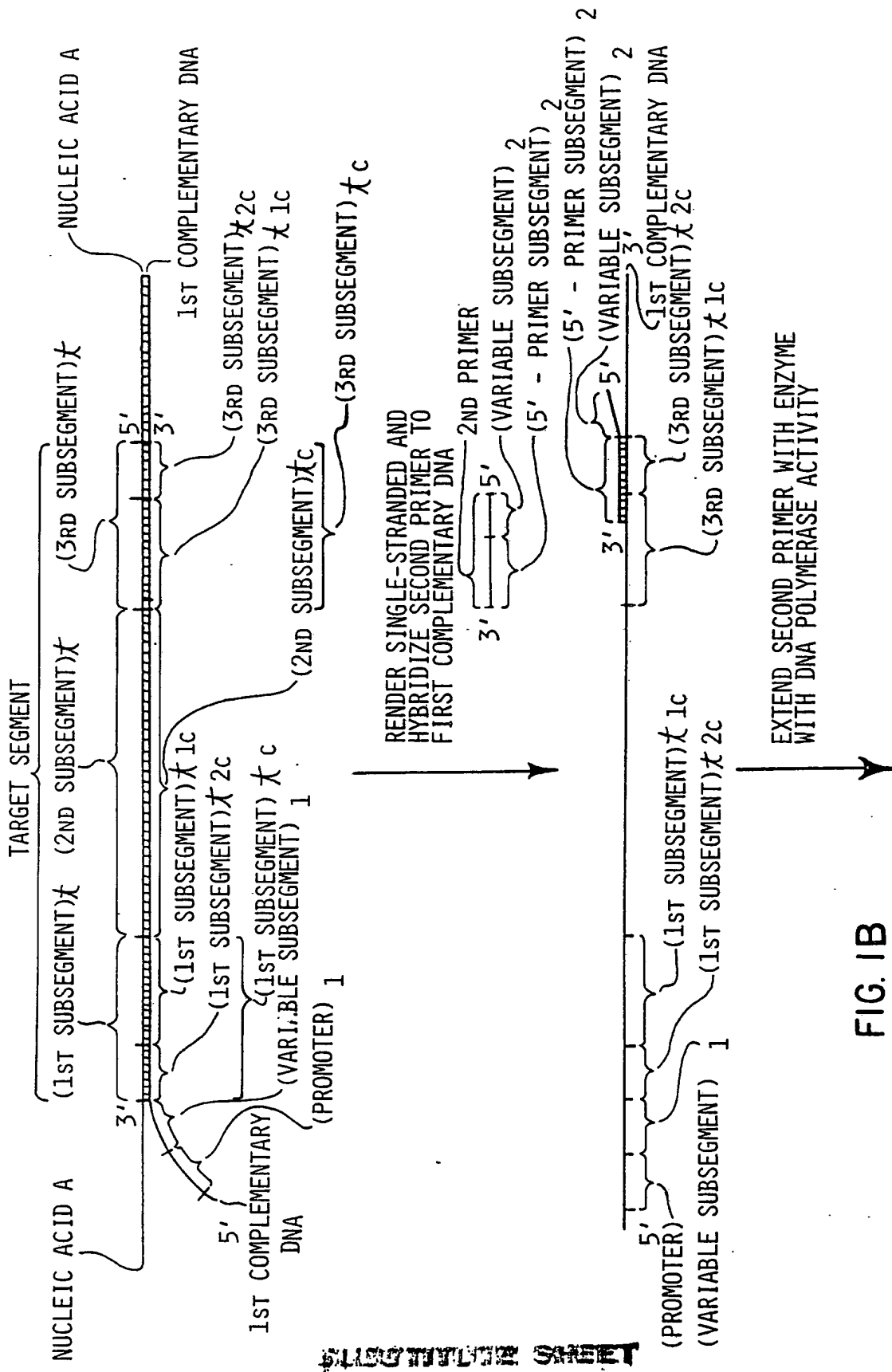


FIG. 1A

Substituted Sheet



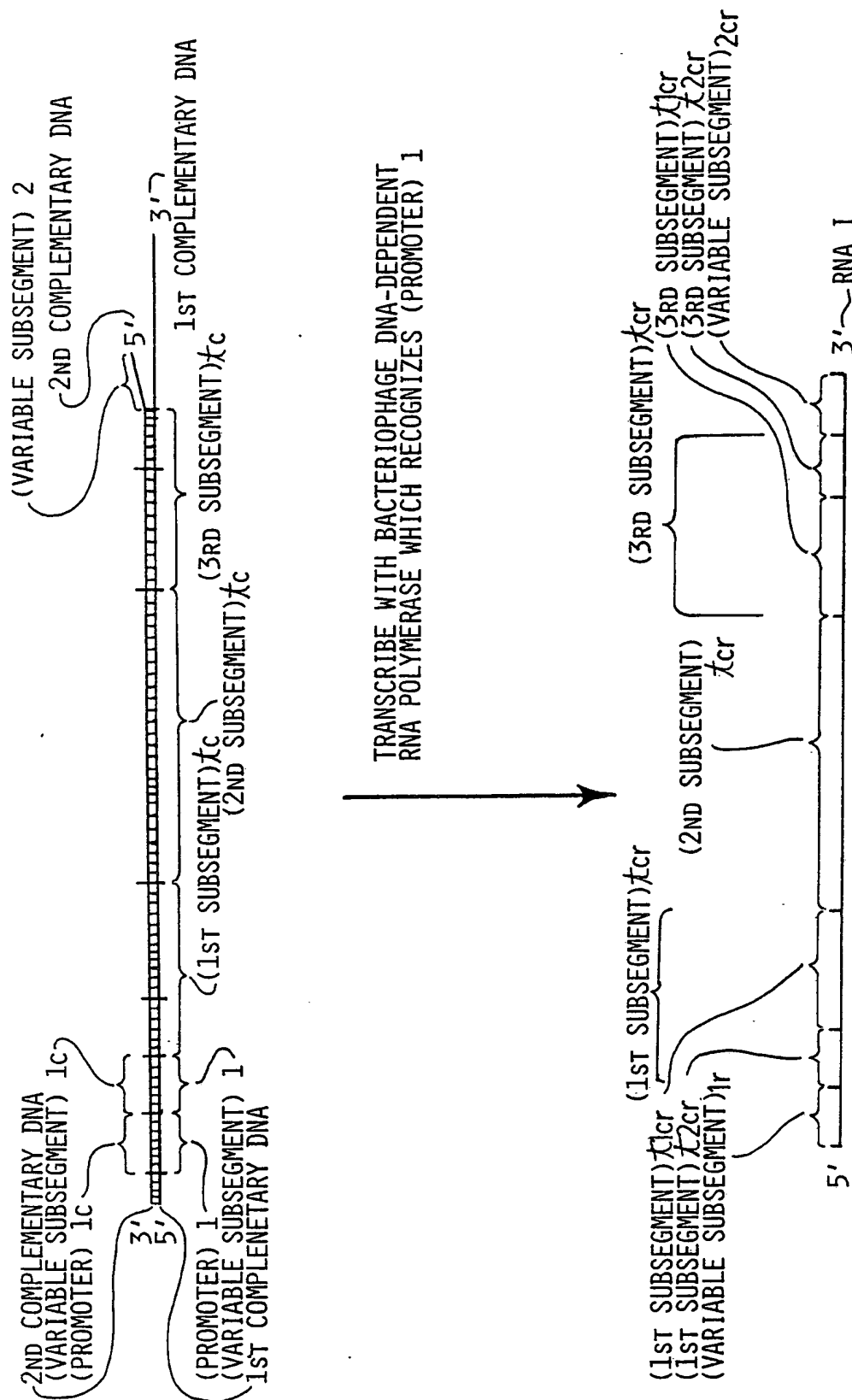


FIG. 1C

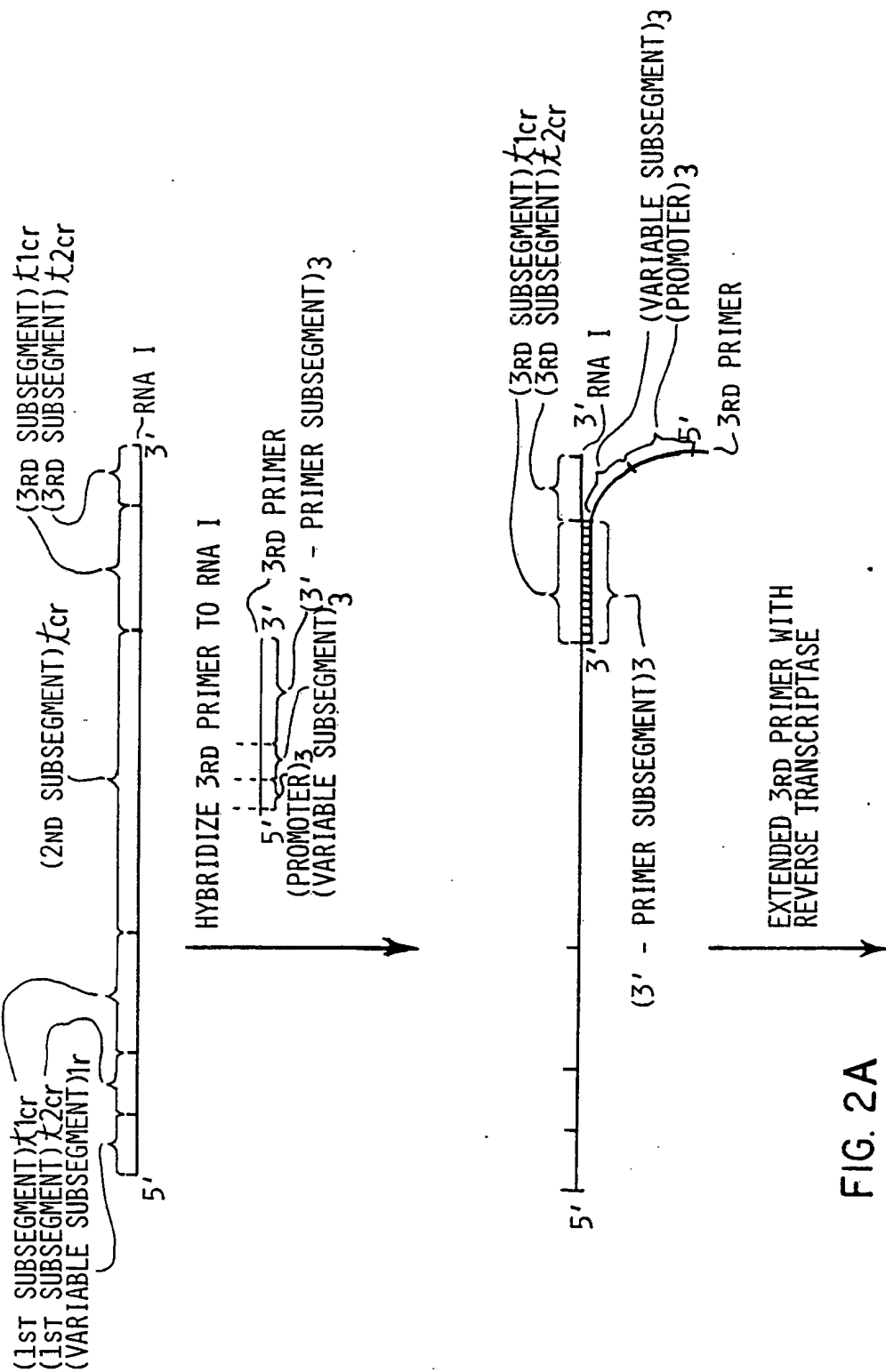


FIG. 2A

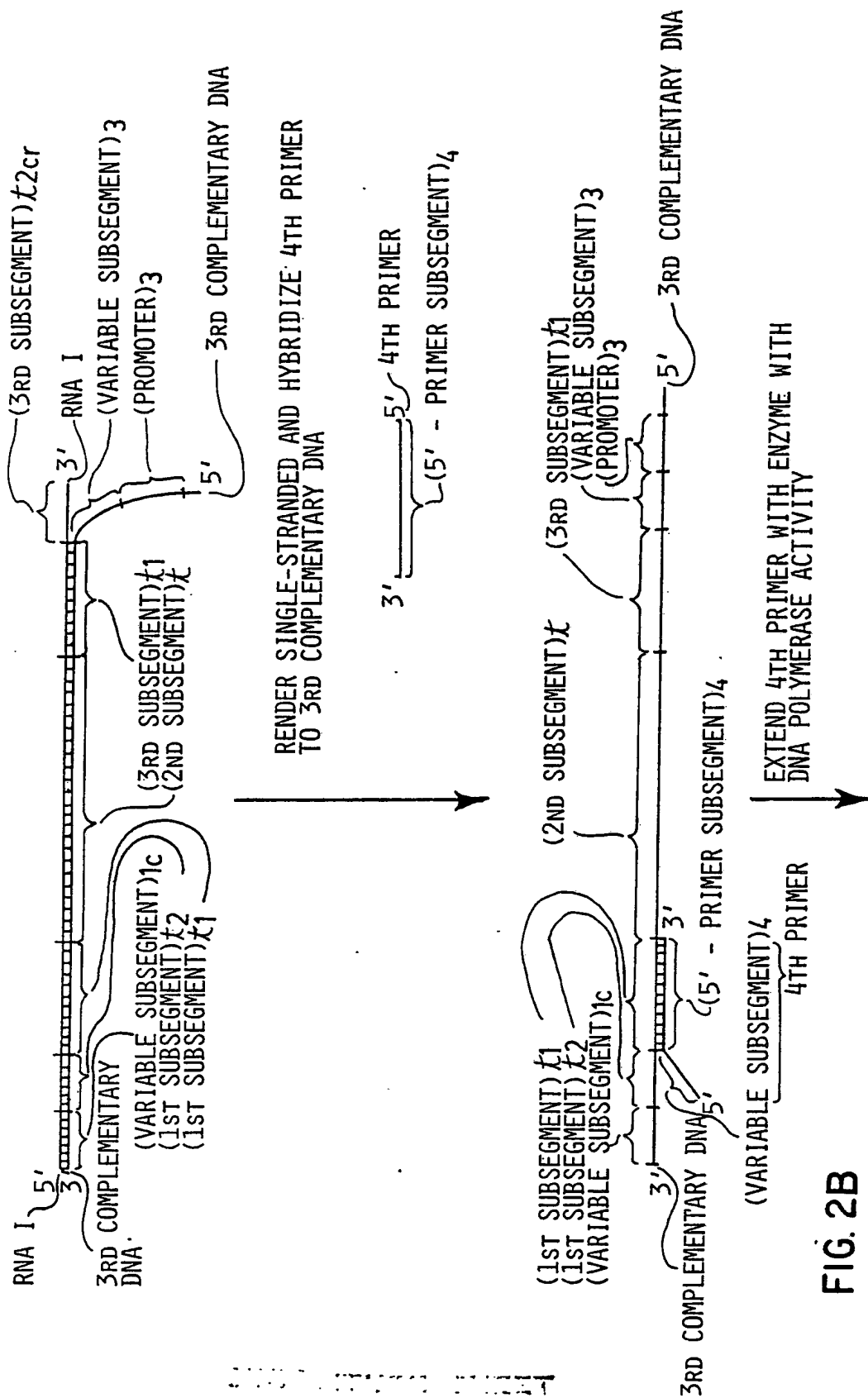


FIG. 2B

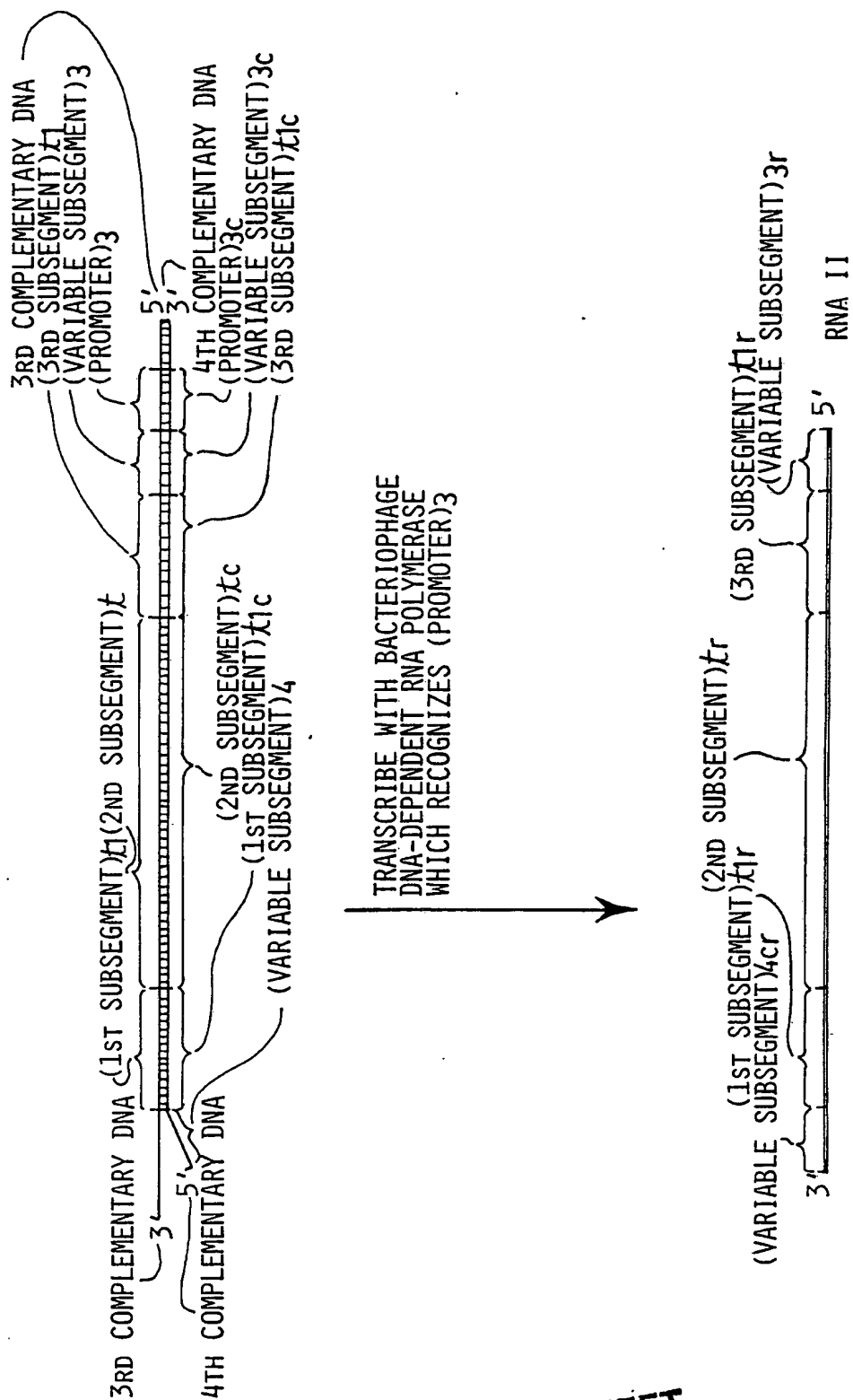


FIG. 2C

REPLACEMENT SHEET

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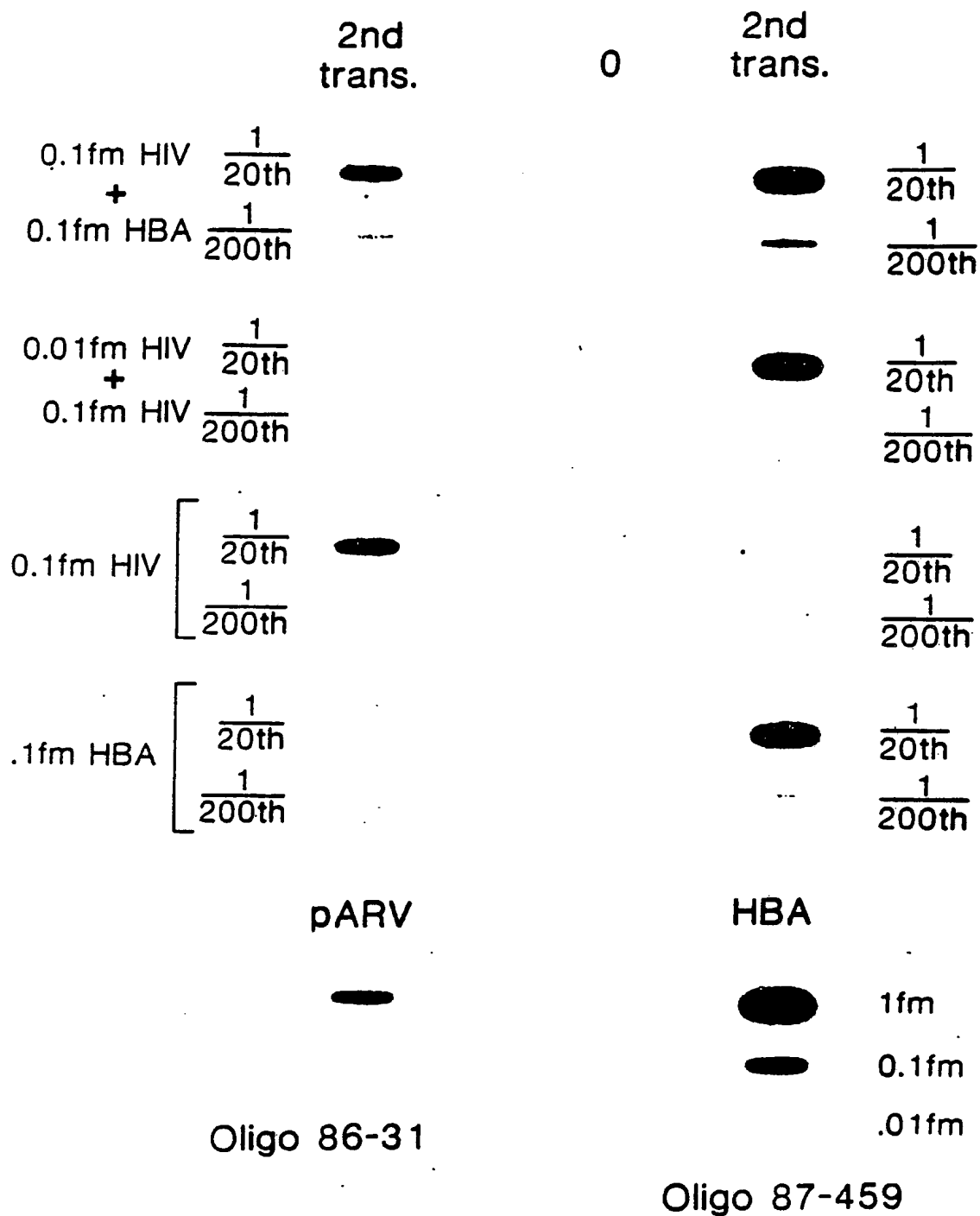
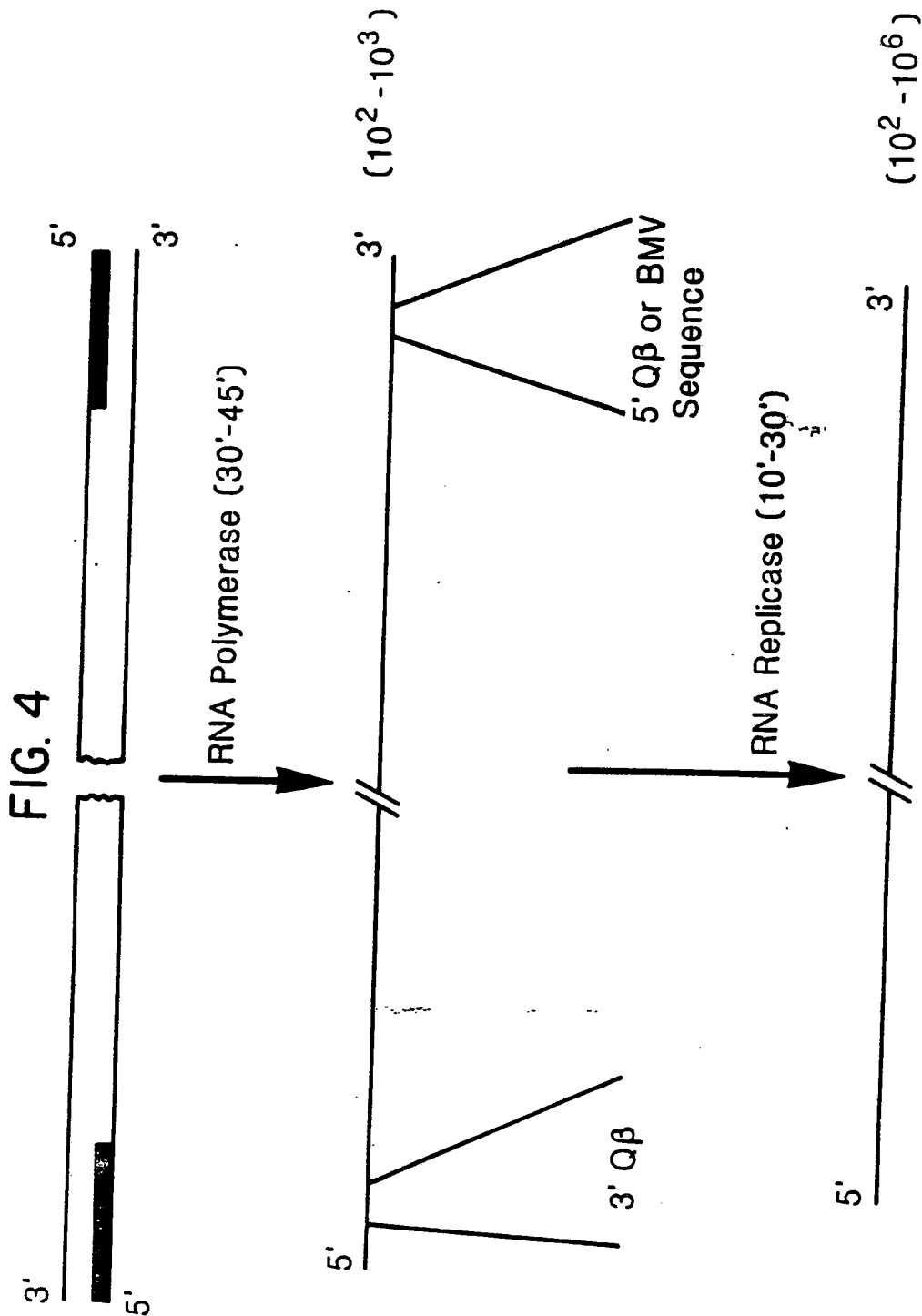



FIG. 3



INTERNATIONAL SEARCH REPORT

International Application No **PCT/US88/02108**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 12 Q 1/68, C 12 N 15/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 12 Q; C 12 N; C 12 P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	Science, Vol. 239, p. 491-493, 29 January 1988 (E.S. STOFLET et al.): "Genomic Amplification with Transcript Sequencing" --	1-51
A	Nucleic Acids Research, Vol. 15, p. 8783-98, October 1987 (J.F. MILLIGAN et al.): "Oligoribonucleotid Synthesis using T7 RNA polymerase and synthetic DNA templates" --	1-51
A	Science, Vol. 230, p. 1350-5, 20 December 1985 (R.K. SAIKI et al.): "Enzymatic Amplification of B-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia" --	1-51
A	EP, A2, 0 201 184 (CETUS CORPORATION) 17 December 1986, whole document --	1-51
./.		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
19th October 1988	16 NOV 1988	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0 200 362 (CETUS CORPORATION) 10 December 1986 whole document --	1-51
P,A	EP, A2, 0 236 069 (CETUS CORPPRATION) 9 September 1987 p. 36-38 --	1-51
P,A	EP, A2, 0 229 701 (CETUS CORPORATION) 22 July 1987 whole document --	1-51

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/02108
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0201184	12-11-86	EP-A- 0200362	05-11-86
		JP-A- 61274697	04-12-86
		JP-A- 62000281	06-01-87
		US-A- 4683202	28-07-87
		CA-A- 1237685	07-06-88
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		JP-A 61274697	04-12-86
		JP-A- 62000281	06-01-87
		US-A- 4683202	28-07-87
		CA-A- 1237685	07-06-88
		US-A- 4683195	28-07-87
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